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<b>(71) Applicant (for all designated States except US):</b> THE SALK INSTITUTE FOR BIOLOGICAL STUDIES [US/US]; 10010 North Torrey Pines Road, La Jolla, CA 92037 (US).		<b>Published</b> <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
<b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> EVANS, Ronald, M. [US/US]; 1471 Cottontail Lane, La Jolla, CA 92037 (US). SAEZ, Enrique [ES/US]; 1056 Diamond Street, San Diego, CA 92109 (US).			
<b>(74) Agent:</b> REITER, Stephen, E.; Gray Cary Ware & Freidenrich LLP, Suite 1600, 4365 Executive Drive, San Diego, CA 92121 (US).			
<b>(54) Title:</b> FORMULATIONS USEFUL FOR MODULATING EXPRESSION OF EXOGENOUS GENES IN MAMMALIAN SYSTEMS, AND PRODUCTS RELATED THERETO			
<b>(57) Abstract</b> <p>In accordance with the present invention, there are provided various methods for modulating the expression of an exogenous gene in a mammalian subject employing modified ecdysone receptors. Also provided are modified ecdysone receptors, as well as homomeric and heterodimeric receptors containing same, nucleic acids encoding invention modified ecdysone receptors, modified hormone response elements, gene transfer vectors, recombinant cells, and transgenic animals containing nucleic acids encoding invention modified ecdysone receptor.</p>			

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Formulations Useful for Modulating Expression Of  
Exogenous Genes In Mammalian Systems, and Products  
Related Thereto

FIELD OF THE INVENTION

The present invention relates to methods in the field of recombinant DNA technology, and products related thereto. More particularly, the invention relates to 5 methods and products for modulating the expression of exogenous genes in mammalian systems.

BACKGROUND OF THE INVENTION

The steroid/thyroid hormone receptors comprise a superfamily of ligand-dependent transcription factors 10 that play a crucial role in mediating changes in cell fate and function (Evans, R.M., Science 240:889-895 (1988)). The receptors transduce extracellular hormonal signals to target genes that contain specific enhancer sequences referred to as hormone response elements (HREs) 15 Evans, (1988); Green and Chambon, Trends Genet. 4:309-314 (1988); Yamamoto, K.R., Annu. Rev. Genet. 19:209-252 (1985)). Each receptor recognizes its own HRE, assuring that a distinct response is triggered by each hormonal signal. Together the collection of related transcription 20 factors and their cognate response elements provides a unique opportunity to control gene expression.

The DNA binding domain of each member of the steroid/thyroid hormone superfamily of receptors has 66-68 amino acids. Twenty of these, including nine 25 cysteines, are conserved throughout the family. The modular structure of members of this receptor superfamily allows the exchange of homologous domains between receptors to create functional chimeras. This strategy was used to demonstrate that the DNA binding domain is 30 solely responsible for the specific recognition of the

HRE in vivo (Green and Chambon, Nature 325:75-78 (1987); Giguère et al., Nature 330:624-629 (1987); Petkovich et al., Nature 330:444-450 (1987); Kumar et al., Cell 51:941-951 (1987); Umesono et al., Nature 336:262-265 (1988); Thompson and Evans, Proc. Natl. Acad. Sci. U.S.A. 86:3494-3498 (1989) and in vitro (Kumar and Chambon, Cell 55:145-156 (1988)). By analogy with the proposed structure for Xenopus transcription factor IIIA (Miller et al., EMBO J. 4:1609-1614 (1985)), the invariant cysteines are thought to form two "zinc fingers" that mediate the DNA binding function (Hollenberg and Evans, Cell 55:899-906 (1988)). Involvement of these cysteines in Zn(II) coordination is supported by extended X-ray absorption fine structure (Freedman et al., Nature 334:543-546 (1988)), and by the effect of point mutagenesis experiments on DNA binding (Hollenberg and Evans, (1988)); Severne et al., EMBO J. 7:2503-2508 (1988)).

The HREs are in fact structurally related but functionally distinct. The glucocorticoid receptor response element (GRE), estrogen receptor response element (ERE), and thyroid hormone receptor response element (TRE) have been characterized in detail. These particular response elements have been found to have a palindromic pair of hexameric "half-sites" (Evans, (1988); Green and Chambon, (1988)). With optimized pseudo- or consensus response elements, only two nucleotides per half-site differ between GRE and ERE (Klock et al., Nature 329:734-736 (1987)). On the other hand, EREs and TREs have identical half-sites but the number of nucleotide spacers between the two half sites is different (Glass et al., Cell 54:313-323 (1988)).

In contrast to response elements having the palindromic sequence motif, the following hormone receptors typically recognize response elements having

two half-sites in a direct-repeat (DR) sequence motif: RXR, RAR, COUP-TF, PPAR, and the like (see, e.g., Mangelsdorf et al., The Retinoids: Biology, Chemistry, and Medicine, 2nd Edition, Raven Press, Ltd., New York, 5 1994, Chapter 8). Thus at least three distinct means are used to achieve HRE diversity: 1) binding site specificity for a particular half-site; 2) nucleotide spacing between the two half-sites; and 3) the orientation of the half-sites to one another.

10 In insect systems, a pulse of the steroid hormone ecdysone triggers metamorphosis in Drosophila melanogaster showing genomic effects, such as chromosomal puffing, within minutes of hormone addition. Mediating this response in insects is the functional 15 ecdysone receptor, a heterodimer of the ecdysone receptor (EcR) and the product of the ultraspiracle gene (USP) (Yao et al. (1993) Nature 366:476-479; and Yao et al. (1992) Cell 71:63-72). Responsiveness to an insect ecdysteroid can be recreated in cultured mammalian cells 20 by co-transfection of EcR, USP, an ecdysone responsive reporter, and treatment with ecdysone or the synthetic analog muristerone A.

The ability to manage the expression of genes introduced into mammalian cells and animals would further 25 advance many areas of biology and medicine. For instance, methods that allow the intentional manipulation of gene expression would facilitate the analysis of genes whose gene products cannot be tolerated constitutively or at certain stages of development. Such methods would 30 also be valuable for clinical applications such as gene therapy protocols, where the expression of a therapeutic gene must be regulated in accordance with the needs of the patient (Saez et al. (1997) Current Opinion in Biotechnology 8:608-616). However, to be of broad 35 benefit, gene regulation techniques must allow for rapid,

robust and precise induction/repression of gene activity. Precise control of gene expression is an invaluable tool in studying, manipulating and controlling development and other physiological processes.

5         Early designs to direct gene expression in mammals were based on endogenous elements, such as cytokine response elements or heat-shock proteins. Due to a high level of basal expression in the uninduced state, and pleiotropic effects brought about by general 10 inducing agents, these systems lack the specificity required to regulate genes in mammalian cells and organisms. (Saez et al., supra)

As another means for controlling gene expression in a mammalian system, an inducible 15 tetracycline regulated system has been devised and utilized in transgenic mice, whereby gene activity is induced in the absence of the antibiotic and repressed in its presence (see, e.g, Gossen et al. (1992) Proc. Natl. Acad. Sci. **89**:5547-5551; Gossen et al. (1993) TIBS **18**:471-475; Furth et al. (1994) Proc. Natl. Acad. Sci. **91**:9302-9306; and Shockett et al. (1995) Proc. Natl. Acad. Sci. **92**:6522-6526). However, problems were noticed during the development of this system including toxicity of the tetracycline transactivator protein, the 25 requirement for continuous treatment of tetracycline to repress expression, and the slow clearance of antibiotic from bone which interferes with quick and precise induction. While this system has been improved by the recent identification of a mutant tetracycline repressor 30 which acts conversely as an inducible activator, the pharmacokinetics of tetracycline may hinder its use during development when a precise and efficient "on-off" switch is essential (Gossen et al. (1995) Science **268**:1766-1769).

Another approach to regulate gene expression relies on induction of protein dimerization, a method derived from studies on the mechanism of action of immunosuppressive agents (Spencer, DM, (1996) Trends Genet, 12:181-187). Compounds such as FK506 and cyclosporin A (CsA) subdue the immune response by binding with high affinity to the immunophilins FKBP12 and cyclophilin (CyP), respectively. Using a synthetic homodimer of FK506 (called FK10102), a general strategy was devised to bring together any two peptides, by endowing them with the domain of FKBP12 to which FK506 binds. By chemically linking FK506 and CsA, a heterodimer molecule that can selectively connect two different immunophilin domains and their respectively attached peptides was also generated.

The resulting heterodimerizer, FKC<sub>s</sub>A, has been used to reconstitute a functional transcription factor by joining a GAL4 DNA-binding domain fused to FKBP12, and the transactivation moiety of VP16 bound to CyP. In cells expressing these chimeric proteins, the expression of a promoter containing GAL4 binding sites was strongly stimulated in the presence of this 'chemical inducer of dimerization' (CID) FKC<sub>s</sub>A.

The immunosuppressive drug rapamycin is a natural heterodimerizer that complexes with FKBP12 and FKBP12-rapamycin-associated protein (FRAP). A new inducible system based on rapamycin builds on the modularity of mammalian transcription factors and the heterodimerizing properties of this drug (Rivera et al. (1996), Nat Med 2:1028-1032). Unfortunately, the attractive pharmacokinetics of this drug are compromised by its effects on the immune system, i.e., rapamycin is incapable of regulating gene expression at doses that are not immunosuppressive.

Two gene control systems based on components of mammalian steroid hormone receptors have recently been developed (Wang et al. (1994) PNAS USA 91:8180-8184, Delort and Capecchi (1996) Num Gene Ther 7:809-820).

5      Created independently, these two steroid-based methods are nonetheless virtually identical, i.e., both methods combine a truncated form of the progesterone receptor hormone-binding domain with a yeast GAL4 DNA-binding moiety, and the transactivation domain of the VP16 10     protein. The mutated progesterone receptor moiety fails to bind progesterone but it retains the ability to bind the progesterone and glucocorticoid antagonist mifepristone (RU486), such that in the presence of RU486, the fusion protein (called either GLVP or TAXI) activates 15     transcription through a multimer of the GAL4 DNA-binding site placed upstream of a minimal promoter.

Although these systems represent an improvement over previous hormone based designs, their performance in cells remains poor. In transient and stable 20     transfections of various cell types, a high level of basal activity dampens the level of inducibility of the above-described approaches. Moreover, since RU486 is an abortifacient, these systems are not likely to be useful for developmental studies. Furthermore, the utility of 25     these approaches for long-term protocols is a concern as the response to RU486 diminishes over time. Experiments with these systems have also hinted at the possibility that these chimeric proteins may interfere with endogenous factors, an observation that would explain why 30     it was difficult to generate transgenics that express these proteins. In spite of these issues, an important advantage of steroid-based systems is that they appear to have more favorable kinetics than tetracycline systems, i.e., lipophilic hormones employed by steroid-based 35     systems are quickly metabolized and have short half-lives in vivo.

Accordingly, there remains a need in the art for improved methods to precisely modulate the expression of exogenous genes in mammalian subjects, as well as expanding the range of formulations which can be used to 5 modulate such systems.

#### BRIEF DESCRIPTION OF THE INVENTION

In accordance with the present invention, there are provided various methods and formulations useful for modulating the expression of exogenous gene(s) in 10 mammalian subject(s). Invention methods and formulations are useful in a wide variety of applications where inducible in vivo expression of exogenous gene(s) is desired, such as in vivo therapeutic methods for 15 delivering recombinant proteins into a variety of cells within a patient.

Unlike prior art tetracycline based strategies, transferring ecdysone responsiveness to mammalian cells takes advantage of a naturally evolved steroid inducible system. Advantages of ecdysteroid use include the 20 lipophilic nature of the compounds (which provides efficient penetration thereof into all tissues, including the brain), short half-lives (which allow for precise and potent inductions), and favorable pharmacokinetics that prevent storage and expedite clearance.

25

#### BRIEF DESCRIPTION OF THE FIGURES

Figures 1A - 1D show the effect on ecdysone responsiveness of using various combinations of USP or RXR with different modified EcRs. In Figure 1A, the numerical values on both sides of the figure are on 30 different scales, with the left half presenting activity in 10 unit increments and the right half presenting reporter activity in 1000 unit increments. Darkened and

stripped bars represent reporter activity with no hormone or 1 $\mu$ M muristerone A (MurA), respectively.

Figure 1B shows FXR and VpEcR activity on ecdysone response element (EcRE) and a hybrid 5 ecdysone/glucocorticoid response element (E/GRE) responsive reporters. VpEcR, VgEcR, and control transfection without receptors were treated with 1 $\mu$ M muristerone. FXR transfections were treated with 50 $\mu$ M Juvenile Hormone III (Sigma). Darkened and stripped bars 10 represent reporter activity with no hormone or 1 $\mu$ M muristerone A/50 $\mu$ M Juvenile Hormone III, respectively.

Figure 1C shows that E/GRE and GRE are non-overlapping response elements. Darkened and stripped bars represent reporter activity with no hormone or 1 $\mu$ M 15 muristerone A/1 $\mu$ M dexamethasone, respectively.

Figure 1D shows a schematic diagram of modified ecdysone receptors. GEcR is a chimeric receptor containing the N-terminal transactivation domain of GR and the DNA- and ligand-binding domains of EcR. VpEcR is 20 an N-terminal truncation of EcR fused to the activation domain of Vp16. VgEcR is identical to VpEcR except for the following point mutations in the P box of the DNA binding domain: E282G, G283S, and G286V. Vp16-EcR-B1 is a fusion of full length EcR with the activation domain of 25 Vp16, wherein the activation domain of Vp16 is fused thereto at the carboxy terminus thereof. VgEcR-B1 is identical to Vp16-EcR-B1 except for the same point mutations in the P box of the DNA binding domain as described above. In Figure 1D, DBD=DNA binding domain 30 and LBD=ligand binding domain.

Figure 2 shows a schematic diagram of an invention ecdysone inducible gene expression system. After expression of RXR and a modified EcR, the two

receptors can heterodimerize and transactivate the ecdysone response element-containing promoter in the presence of hormone. The ecdysone response elements are placed upstream of a minimal promoter (i.e., an enhancerless promoter) which can drive the expression of any exogenous cDNA.

Figure 3 shows the dose-dependent activation of CV-1 cells with muristerone A alone (●), an RXR ligand (6-(1-(3,5,5,8,8-pentamethyl-5,6,7,8-tetrahydronaphthalen-2-yl)cyclopropyl)nicotinic acid (LG1000268)) alone (◆), or a combination of the two (MurA and LG1000268; ■), where the concentration of MurA is varied while the concentration of LG1000268 is maintained constant (100nM).

Figure 4 shows the dose-dependent activation of CV-1 cells with N'-(3,5-dimethylbenzoyl)-N-(2-methyl-3,4-(ethylenedioxy)-benzoyl)-N'-(tert-butyl) hydrazine alone (●) or in the further presence of (6-(1-(3,5,5,8,8-pentamethyl-5,6,7,8-tetrahydronaphthalen-2-yl)cyclopropyl)nicotinic acid (LG1000268), ■), N'-(3,5-dimethylbenzoyl)-N-(4-ethylbenzoyl)-N'-(tert-butyl) hydrazine alone (▲) or in the further presence of LG1000268 (◆).

Figure 5 shows the dose-dependent activation of CV-1 cells with ponasterone A (ponA) alone, or in combination with the RXR agonists, LG1000268 ((6-(1-(3,5,5,8,8-pentamethyl-5,6,7,8-tetrahydronaphthalen-2-yl)cyclopropyl)nicotinic acid), LGD1069 (4-(1-(3,5,5,8,8-pentamethyl-5,6,7,8-tetrahydro-2-naphthyl)-ethenyl)benzoic acid) and 9-cis-RA (9-cis-retinoic acid).

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention, there are provided methods for modulating the expression of an exogenous gene in a mammalian subject containing:

5 (i) a DNA construct comprising said exogenous gene operatively associated with a response element; and

10 (ii) a modified ecdysone receptor which, in the optional presence of a silent partner therefor, is capable of binding to said response element;

said method comprising administering to said subject an effective amount of a first ligand and a second ligand,

15 wherein said first ligand is:

a ligand for said modified ecdysone receptor, not normally present in the cells of said subject, and  
not toxic to said subject; and

20 wherein said second ligand is a ligand for said silent partner.

Thus, in accordance with the present invention the insect molting hormone, ecdysone (as well as analogs and mimics thereof), is advantageously employed as a 25 regulatable inducer of gene expression in mammalian systems, i.e., background levels of expression are substantially zero in the absence of conditions required for induction. In a presently preferred aspect of the invention, promoters operatively associated with novel 30 modified ecdysone response element(s) are employed in conjunction with modified ecdysone receptor(s)

(preferably having altered DNA binding specificity) to provide an extremely powerful and specific inducible mammalian expression system. The low basal activity of the invention expression system is advantageously 5 suitable for the expression of transcription factors and toxic genes. The excellent dose response and induction rate characteristics of the invention inducible expression system allow for precise control of both the degree and duration of induction of a desired gene.

10 Since the invention method provides for regulated gene expression by an exogenous non-mammalian inducer, it can be advantageously employed in a variety of in vivo, ex vivo and in vitro mammalian expression systems. For example, inducible expression of flp 15 recombinase in transgenic mammals, in accordance with invention methods, would enable those of skill in the art to accomplish temporally specific inducible gene targeting of the adult or the developing embryo (See, e.g., U.S. Patent Nos. 5,654,182 and 5,677,177).

20 As employed herein, the terms "modulate" and "modulating" refer to the ability of a given ligand/receptor complex to effect transactivation of transcription of an exogenous gene, relative to such ability of said receptor in the absence of ligand. The 25 actual effect of complex formation on the transactivation activity of a receptor will vary depending on the specific receptor species which are part of the ligand/receptor complex, and on the response element with which the ligand/receptor complex interacts.

30 As used herein, when referring to genes, the phrase "exogenous to said mammalian subject" or simply "exogenous" refers to any gene wherein the gene product is not naturally expressed in the particular cell where expression is desired. For example, exogenous genes can

be either natural, i.e., wild type, or synthetic genes and therapeutic genes, which are introduced into the subject in the form of DNA or RNA. The gene of interest can be introduced into target cells (for in vitro applications), or the gene of interest can be introduced directly into a subject, or indirectly introduced by the transfer of transformed cells into a subject. When referring to ligands, inducers or activators, the term "exogenous" refers to compounds which originate or are produced outside the particular cell or organism where expression is desired.

"Wild type" genes are those that are native to cells of a particular type. Wild type genes contemplated for use in the practice of the present invention include genes which encode a gene product:

the substantial absence of which leads to the occurrence of a non-normal state in said subject; or

20 a substantial excess of which leads to the occurrence of a non-normal state in said subject;

and the like.

Such genes may not be expressed in biologically significant levels or may be undesirably overexpressed, 25 respectively, to result in a non-normal state, such as diseases, injuries, maladies, neoplasms, disorders, infections, and the like. Thus, for example, while a synthetic or natural gene encoding human insulin would be exogenous genetic material to a yeast cell (since yeast 30 cells do not naturally contain insulin genes), a human insulin gene inserted into a human skin fibroblast cell would be a wild type gene with respect to the fibroblast since human skin fibroblasts contain genetic material encoding human insulin, although human skin fibroblasts 35 do not express human insulin in biologically significant levels.

As employed herein, the phrase "therapeutic gene" refers to a gene which imparts a beneficial function to the host cell in which such gene is expressed. Therapeutic genes are those that are not naturally found in host cells. For example, a synthetic or natural gene coding for wild type human insulin would be therapeutic when inserted into a skin fibroblast cell so as to be expressed in a human host, where the human host is not otherwise capable of expressing functionally active human insulin in biologically significant levels. In accordance with the methods described herein, therapeutic genes are expressed at a level that provides a therapeutically effective amount of the corresponding therapeutic protein.

Therapeutic genes contemplated for use in the practice of the present invention include genes which encode a gene product:

which is toxic to the cells in which it is expressed; or

which imparts a beneficial property to the host subject (e.g., disease resistance, etc); and the like.

Numerous genomic and cDNA nucleic acid sequences coding for a variety of proteins are well known in the art. Exogenous genetic material useful in the practice of the present invention include genes that encode biologically active proteins of interest, such as, e.g., secretory proteins that can be released from said cell; enzymes that can metabolize a substrate from a toxic substance to a non-toxic substance, or from an inactive substance to a useful substance; regulatory proteins; cell surface receptors; and the like. Useful genes include genes that encode blood clotting factors such as human factors VIII and IX; genes that encode hormones such as insulin, parathyroid hormone,

luteinizing hormone releasing factor (LHRH), alpha and beta seminal inhibins, and human growth hormone; genes that encode proteins such as enzymes, the absence of which leads to the occurrence of an abnormal state; genes 5 encoding cytokines or lymphokines such as interferons, granulocytic macrophage colony stimulating factor (GM-CSF), colony stimulating factor-1 (CSF-1), tumor necrosis factor (TNF), and erythropoietin (EPO); genes encoding inhibitor substances such as alpha<sub>1</sub>-antitrypsin; genes 10 encoding substances that function as drugs, e.g., genes encoding the diphtheria and cholera toxins; and the like.

Typically, nucleic acid sequence information for a desired protein can be located in one of many public access databases, e.g., GENBANK, EMBL, Swiss-Prot, 15 and PIR, or in many biology related journal publications. Thus, those of skill in the art have access to nucleic acid sequence information for virtually all known genes. Those of skill in the art can either obtain the corresponding nucleic acid molecule directly from a 20 public depository or the institution that published the sequence. Optionally, once the nucleic acid sequence encoding a desired protein has been ascertained, the skilled artisan can employ routine methods, e.g., polymerase chain reaction (PCR) amplification, to isolate 25 the desired nucleic acid molecule from the appropriate nucleic acid library. Thus, all known nucleic acids encoding proteins of interest are available for use in the methods and products described herein.

As used herein, the terms "mammal" and 30 "mammalian" refer to humans; domesticated animals, e.g., rats, mice, rabbits, canines, felines, and the like; farm animals, e.g., chickens, cows, pigs and sheep, and the like; and animals of zoological interest, e.g., monkeys and baboons, and the like.

Modified ecdysone receptors contemplated for use in the practice of the present invention comprise:

5 a ligand binding domain capable of binding an ecdysteroid;

10 a DNA-binding domain, preferably obtained from a DNA-binding protein; and

an activation domain, preferably of a transcription factor,

15 wherein at least one of said DNA-binding domain or said activation domain is not obtained from a native ecdysone receptor.

Preferably, when said activation domain is derived from a glucocorticoid receptor, said DNA-binding domain is not derived from a glucocorticoid receptor or an E. coli LexA 20 protein. In accordance with the present invention, modified ecdysone receptors function in expression systems, preferably mammalian, to transactivate gene expression from transcription regulatory regions having ecdysone response elements. Preferably, in order to minimize induction of undesired gene expression, modified ecdysone receptors of the invention will have substantially no constitutive activity in mammalian cells.

25 Ligand binding domains capable of binding an ecdysteroid, as contemplated for use in the preparation of invention modified ecdysone receptors are typically derived from the carboxy-terminal portion of native ecdysone receptor (see, for example, Koelle et al., Cell, 67:59-77, 1991; Christopherson et al., PNAS, USA, 30 89:6314-6318, 1992; Jepson et al. WO 96/37609, 1996).

Ligand binding domains capable of binding an ecdysteroid can be functionally located in either orientation and at various positions within the modified ecdysone receptor of the invention. For example, the ligand binding domain 35 capable of binding an ecdysteroid can be positioned at either the amino or carboxy terminus of the modified

receptor, or therebetween. In a preferred embodiment of the present invention, the ligand binding domain capable of binding an ecdysteroid is positioned at the carboxy terminus of the modified receptor (see Figure 1).

5 DNA-binding domains contemplated for use in the preparation of modified ecdysone receptors contemplated for use in the practice of the present invention are typically obtained from DNA-binding proteins (e.g., transcription factors). The term "DNA-binding domain" is  
10 understood in the art to refer to an amino acid sequence that is able to bind to DNA. As used herein, the term "DNA-binding domain" encompasses a minimal peptide sequence of a DNA-binding protein, up to the entire length of a DNA-binding protein, so long as the DNA-  
15 binding domain functions to associate with a particular response element.

Such DNA-binding domains are known to function heterologously in combination with other functional protein domains by maintaining the ability to bind the  
20 natural DNA recognition sequence (see, e.g., Brent and Ptashne, 1985, Cell, 43:729-736). For example, hormone receptors are known to have interchangeable DNA-binding domains that function in chimeric proteins (see, e.g., U.S. Patent No. 4,981,784; and Evans, R., 1988, Science,  
25 240:889-895). Thus, similar to the ligand binding domain of invention modified ecdysone receptor, the DNA-binding domain can be positioned at either the carboxy terminus or the amino terminus, or the DNA-binding domain can be positioned between the ligand binding domain and the  
30 activation domain. In preferred embodiments of the present invention, the DNA-binding domain is positioned internally between the ligand binding domain and the activation domain.

"DNA-binding protein(s)" contemplated for use herein belong to the well-known class of proteins that are able to directly bind DNA and facilitate initiation or repression of transcription. Exemplary DNA-binding 5 proteins contemplated for use herein include transcription control proteins (e.g., transcription factors and the like; Conaway and Conaway, 1994, "Transcription Mechanisms and Regulation", Raven Press Series on Molecular and Cellular Biology, Vol. 3, Raven 10 Press, Ltd., New York, NY).

Transcription factors contemplated for use herein as a source of such DNA binding domains include, e.g., homeobox proteins, zinc finger proteins, hormone receptors, helix-turn-helix proteins, helix-loop-helix 15 proteins, basic-Zip proteins (bZip),  $\beta$ -ribbon factors, and the like. See, for example, Harrison, S., "A Structural Taxonomy of DNA-binding Domains," Nature, 353:715-719. Homeobox DNA-binding proteins suitable for use herein include, for example, HOX, STF-1 (Leonard et 20 al., 1993, Mol. Endo., 7:1275-1283), Antp, Mat  $\alpha$ -2, INV, and the like. See, also, Scott et al. (1989), Biochem. Biophys. Acta, 989:25-48. It has been found that a fragment of 76 amino acids (corresponding to amino acids 140-215 described in Leonard et al., 1993, Mol. Endo., 25 7:1275-1283) containing the STF-1 homeodomain binds DNA as tightly as wild-type STF-1. Suitable zinc finger DNA-binding proteins for use herein include Zif268, GLI, XFin, and the like. See also, Klug and Rhodes (1987), Trends Biochem. Sci., 12:464; Jacobs and Michaels (1990), 30 New Biol., 2:583; and Jacobs (1992), EMBO J., 11:4507-4517.

An additional DNA binding domain contemplated for use in the practice of the present invention is the GAL4 DNA binding domain. The DNA binding domain of the 35 yeast GAL4 protein comprises at least the first 74 amino

terminal amino acids thereof (see, for example, Keegan et al., Science 231:699-704 (1986)). Preferably, the first 90 or more amino terminal amino acids of the GAL4 protein will be used, with the 147 amino terminal amino acid 5 residues of yeast GAL4 being presently most preferred.

Preferably, the DNA-binding domain used herein is obtained from a member of the steroid/thyroid hormone superfamily of receptors. As used herein, the phrase "member(s) of the steroid/thyroid hormone superfamily of 10 receptors" (also known as "nuclear receptors" or "intracellular receptors") refers to hormone binding proteins that operate as ligand-dependent transcription factors, including identified members of the steroid/thyroid hormone superfamily of receptors for 15 which specific ligands have not yet been identified (referred to hereinafter as "orphan receptors").

Exemplary members of the steroid/thyroid hormone superfamily of receptors (including the various isoforms thereof) include steroid receptors such as 20 glucocorticoid receptor (GR), mineralocorticoid receptor (MR), estrogen receptor (ER), progesterone receptor (PR), androgen receptor (AR), vitamin D<sub>3</sub> receptor (VDR), various isoforms of peroxisome proliferator-activated receptors (PPARs), and the like; plus retinoid receptors, such as 25 the various isoforms of retinoic acid receptor (e.g., RAR $\alpha$ , RAR $\beta$ , or RAR $\gamma$ ), the various isoforms of retinoid X receptor (e.g., RXR $\alpha$ , RXR $\beta$ , or RXR $\gamma$ ), and the like (see, e.g., U.S. Patent Nos. 4,981,784; 5,171,671; and 30 5,071,773); thyroid receptors (TR), such as TR $\alpha$ , TR $\beta$ , and the like; insect derived receptors such as the ecdysone receptor, and the like; as well as other gene products which, by their structure and properties, are considered to be members of the superfamily, as defined hereinabove, including the various isoforms thereof. Examples of 35 orphan receptors contemplated for use herein as a source

of DNA binding domain include HNF4 (see, for example, Sladek et al., in Genes & Development 4: 2353-2365 (1990)), the COUP family of receptors (see, for example, Miyajima et al., in Nucleic Acids Research 16: 11057-11074 (1988), and Wang et al., in Nature 340: 163-166 (1989)), COUP-like receptors and COUP homologs, such as those described by Mlodzik et al., in Cell 60: 211-224 (1990) and Ladias et al., in Science 251: 561-565 (1991), the insect derived knirps and knirps-related receptors, and the like.

The DNA-binding domains of all members of the steroid/thyroid hormone superfamily of receptors are related, consisting of 66-68 amino acid residues, and possessing about 20 invariant amino acid residues, including nine cysteines. A member of the superfamily can be characterized as a protein which contains these 20 invariant amino acid residues. The highly conserved amino acids of the DNA-binding domain of members of the superfamily are as follows:

Cys - X - X - Cys - X - X - Asp\* - X  
- Ala\* - X - Gly\* - X - Tyr\* - X - X  
- X - X - Cys - X - X - Cys - Lys\* -  
X - Phe - Phe - X - Arg\* - X - X - X  
- X - X - X - X - X - X - (X - X - )  
Cys - X - X - X - X - X - (X - X - X - )  
Cys - X - X - X - Lys - X - X - Arg  
- X - X - Cys - X - X - Cys - Arg\* -  
X - X - Lys\* - Cys - X - X - X - Gly\*  
- Met (SEQ ID NO:1);

wherein X designates non-conserved amino acids within the DNA-binding domain; an asterisk denotes the amino acid residues which are almost universally conserved, but for which variations have been found in some identified hormone receptors; and the residues enclosed in

parenthesis are optional residues (thus, the DNA-binding domain is a minimum of 66 amino acids in length, but can contain several additional residues).

Modification of existing DNA-binding domains to 5 recognize new and/or specific target recognition sequences is also contemplated herein. For example, in accordance with the present invention, it has been found that the modification of the "P-box" sequence of DNA-binding domains of members of the steroid/thyroid hormone 10 superfamily of receptors offers unique advantages not present in other chimeric hormone receptors. For example, the modification of a P-box amino acid sequence to preferentially bind to a different hormone response element half-site than the naturally occurring P-box 15 amino acid sequence can reduce undesired background levels of gene expression. Thus, invention receptors and methods provide the advantage of increasing the selectivity of exogenous gene expression in a particular subject.

20 As used herein, the phrase "P-box amino acid sequence" refers to the proximal element region in a DNA-binding domain of a hormone receptor that typically occurs at the junction of the first zinc finger and the linker region, e.g., at about amino acids 19-23 of the 25 DNA-binding domain (i.e., amino acids 19-23 of SEQ ID NO:1; see, e.g., Umesono et al. (1989), Cell, 57:1139-1146, Figure 2). Umesono et al. (1989), supra, in Table 1, describe various naturally occurring P-box amino acid sequences for a variety of hormone receptor DNA-binding 30 domains.

In one embodiment of the present invention, the P-box sequence of a hormone receptor DNA-binding domain is modified to have a P-box amino acid sequence that differs from the naturally occurring P-box amino acid

sequence. In a preferred embodiment of the present invention, the modified P-box amino acid sequence differs from the naturally occurring P-box amino acid sequence by 3 amino acids.

5 Preferably, the P-box amino acid sequence is modified so that only the half-site nucleotide sequence recognized by the DNA-binding domain is changed while not altering the spacing between the two half-sites recognized by the DNA-binding domain. For example, when  
10 the DNA-binding domain of the ecdysone receptor is employed in an invention modified ecdysone receptor, the P-box can be modified from the amino acid sequence EGCKG (SEQ ID NO:2; which recognizes the half-site -AGGTCA-) to the amino acid sequence GSCKV (SEQ ID NO:3; which  
15 recognizes the half-site sequence -AGAACCA-). In a presently preferred embodiment, when the DNA-binding domain of invention modified ecdysone receptor is derived from ecdysone receptor, the P-box amino acid sequence is modified to GSCKV (SEQ ID NO:3).

20 It has also been found that in vitro evolution methods can be applied to modify and improve existing DNA-binding domains (see, e.g., Devlin et al., 1990, Science, 249:404-406; and Scott and Smith, 1990, Science, 249:386-390). Alternatively, DNA-binding domains which  
25 are engineered with novel DNA-recognition specificity (see, e.g., Pomerantz et al. Science 267:93-96, 1995, ZFHD1, an engineered transcription factor with a composite DNA-binding domain) are also contemplated.

Activation domains contemplated for use in the  
30 preparation of invention modified ecdysone receptor are typically derived from transcription factors and comprise a contiguous sequence of amino acids that functions to activate gene expression when associated with a suitable DNA-binding domain and a suitable ligand binding domain.

As with the ligand and DNA-binding domains employed for the preparation of invention modified ecdysone receptors, the activation domain can be positioned at the carboxy terminus, the amino terminus or between the ligand 5 binding domain and the DNA binding domain. In preferred embodiments of present invention, the activation domain is positioned at the amino terminus of the modified ecdysone receptor.

Suitable activation domains can be obtained 10 from a variety of sources, e.g., from the N-terminal region of a member of the steroid/thyroid hormone superfamily of receptors, from a transcription factor activation domain, such as, for example, VP16, GAL4, NF- $\kappa$ B or BP64 activation domains, and the like. The 15 presently most preferred activation domain contemplated for use in the practice of the present invention is obtained from the N-terminal region of the VP16 protein.

The presently most preferred modified ecdysone receptors contemplated for use herein are VgEcR, VpEcR, 20 GEcR, Vp16-EcR-B1 or VgEcR-B1 (see Figure 1). VgEcR, VgEcR-B1, and Vp16-EcR-B1 are the presently most preferred receptors contemplated for use herein. The preparation of several of these modified ecdysone receptors is set forth hereinafter in Example 1. Those 25 modified receptors for which explicit methods of preparation is not provided herein can readily be made using the methodology set forth herein in combination with standard methodology well known to those of skill in the art.

30 Invention modified ecdysone receptor proteins can be produced by expressing nucleic acid constructs encoding the chimeric proteins in suitable host cells as described in Example 1. Recombinant methods of producing desired proteins by introducing an expression construct

into appropriate host cells are well-known in the art. Modified ecdysone receptors of the invention can be introduced into a particular subject by direct introduction of the proteins themselves, by introducing 5 DNA construct(s) encoding the receptor into the subject, or into cells obtained from the subject (wherein the cells are transformed and subsequently returned to the subject).

In a preferred embodiment, invention modified 10 ecdysone receptors are expressed under the control of a tissue specific promoter. As readily understood by those of skill in the art, the term "tissue specific" refers to the substantially exclusive initiation of transcription 15 in the tissue from which a particular promoter drives expression of a given gene.

In accordance with one aspect of the present invention, invention modified ecdysone receptors are present in the form of heterodimeric species comprising an invention modified ecdysone receptor and at least one 20 silent partner of the steroid/thyroid hormone superfamily of receptors. Preferably, the silent partner is a mammalian-derived receptor, with RXR being especially preferred.

Silent partners contemplated herein are members 25 of the steroid/thyroid hormone superfamily of receptors which are capable of forming heterodimeric species with the invention modified ecdysone receptor, wherein the silent partner does not directly participate in binding the first ligand (i.e., the ecdysteroid receptor binds 30 the first ligand) but enhances or synergizes ecdysteroid receptor transcription activity upon association with the second ligand. The silent partner can either be endogenous to the cells of the subject or can be provided to the subject by introducing DNA construct(s) encoding

receptor into the subject. A preferred silent partner for use herein is RXR. In a particular embodiment of the present invention, exogenous RXR is provided to said mammalian subject in conjunction with or independent of 5 the invention modified ecdysone receptor.

The formation of heterodimeric receptor(s) can modulate the ability of member(s) of the steroid/thyroid hormone superfamily of receptors to trans-activate transcription of genes maintained under expression 10 control in the presence of ligand for the receptor. For example, formation of a heterodimer of the modified ecdysone receptor with another mammalian hormone receptor promotes the ability of the modified ecdysone receptor to induce trans-activation activity in the presence of an 15 ecdysone response element.

In accordance with another aspect of the present invention, invention modified ecdysone receptors are present in the form of homodimeric species comprising a plurality (i.e., at least two) of invention modified 20 ecdysone receptors, wherein the homodimeric species is capable of associating with RXR, preferably endogenous RXR.

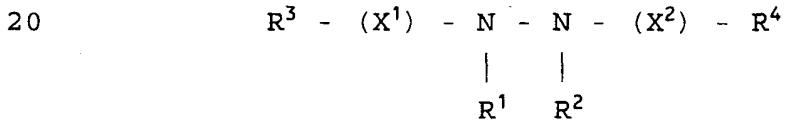
Ligands contemplated for use herein (i.e., the "first" and "second" ligand referred to herein) are 25 compounds which, inside a cell, interact (directly or indirectly) with a homodimer or heterodimer including invention modified ecdysone receptor(s) and silent partner therefor, thereby creating a ligand/receptor complex, which in turn can induce transcription through 30 an appropriate response element. The terms "ecdysone", "ecdysteroid", "ecdysone-analogs", and "ecdysone mimics" as interchangeably used herein, are employed herein in the generic sense (in accordance with common usage in the art), referring to a family of ligands (i.e., first

ligand (also referred herein as "activator," "agonist" or "inducer") with the appropriate transactivation activity (see, for example, Cherbas et al., in Biosynthesis, metabolism and mode of action of invertebrate hormones 5 (ed. J. Hoffmann and M. Porchet), p. 305-322; Springer-Verlag, Berlin). An ecdysone, therefore, is a steroid, steroid-like or non-steroidal compound which acts to modulate gene transcription for a gene maintained under the control of a suitable response element, as described 10 herein.

20-Hydroxy-ecdysone (also known as  $\beta$ -ecdysone) is the major naturally occurring ecdysone. Unsubstituted ecdysone (also known as  $\alpha$ -ecdysone) is converted in peripheral tissues to  $\beta$ -ecdysone. Analogs of the 15 naturally occurring ecdysones are also contemplated within the scope of the present invention. Examples of such analogs, commonly referred to as ecdysteroids, include ponasterone A, ponasterone B, ponasterone C, ponasterone D, 26-iodoponasterone A, muristerone A, 20 inokosterone, 26-mesylinokosterone, sidasterone, buterosterone, ajugasterone, makisterone, cyasterone, sengosterone, and the like. Since it has been previously reported that the above-described ecdysones are neither toxic, teratogenic, nor known to affect mammalian 25 physiology, they are ideal candidates for use as inducers in cultured cells and transgenic mammals according to the invention methods.

Additional compounds contemplated for use herein are mimics of the naturally occurring ecdysones, 30 i.e., synthetic organic compounds which have transactivation activity characteristic of the naturally occurring ecdysones. Examples of such compounds, referred to herein as ecdysone mimics, include 1,2-diacyl hydrazines (e.g., those described in U.S. Patent Nos. 35 5,424,333 and 5,354,762, the entire contents of each of

which are hereby incorporated by reference herein), N'-substituted-N,N'-di-substituted hydrazines (e.g., those described in U.S. Patent No. 5,117,057, the entire contents of which are hereby incorporated by reference herein), dibenzoylalkyl cyanohydrazines (e.g., those described in European Application No. 461,809, the entire contents of which are hereby incorporated by reference herein), N-substituted-N-alkyl-N,N'-diaroyl hydrazines (e.g., those described in U.S. Patent No. 5,225,443, the entire contents of which are hereby incorporated by reference herein), N-substituted-N-acyl-N-alkyl, carbonyl hydrazines (e.g., those described in European Application No. 234,944, the entire contents of which are hereby incorporated by reference herein), N-aroyl-N'-alkyl-N'-aroyl hydrazines (e.g., those described in U.S. Patent No. 4,985,461, the entire contents of which are hereby incorporated by reference herein), and the like. Compounds of specific interest are those having the formula:



wherein:

25  $R^1$  is optionally hydrogen, lower alkyl or substituted lower alkyl, alkenyl or substituted alkenyl, alkynyl or substituted alkynyl, aryl or substituted aryl, heteroaryl or substituted heteroaryl, and the like.  $R^1$  is not present when  $X^1$  is part of a carbon-nitrogen double bond linking  $R^3$  to the hydrazino group.

30  $R^2$  is optionally hydrogen, alkyl or substituted alkyl, cyclohexyl or substituted cyclohexyl, and the like.  $R^2$  is not present when  $X^2$  is part of a carbon-nitrogen double bond linking  $R^4$  to the hydrazino group.

$R^3$  and  $R^4$  are independently part of an appropriately substituted carbon-nitrogen double bond which links  $R^3$  and/or  $R^4$  to the hydrazino linkage, or  $R^3$  and  $R^4$  are independently aryl or substituted aryl,

5 heteroaryl or substituted heteroaryl, provided, however, that when two adjacent positions on the aryl or heteroaryl moieties are substituted with alkoxy, thioalkyl, alkylamino, or dialkylamino groups, these groups may be joined to form a 5- or 6- membered

10 heterocyclic ring system, or  $R^3$  and  $R^4$  are independently heterocyclic or substituted heterocyclic, cycloalkyl or substituted cycloalkyl, and the like.

$X^1$  and  $X^2$  are independently  $-C(O)-$ ,  $-C(S)-$ ,  $-C(NR_2)-$ ,  $-C(=CN)NH-$ ,  $-C(O)O-$ ,  $-C(O)NH-$ ,  $-C(O)NHSO_2-$ ,

15  $-CH_2-$ ,  $-SO_2-$ ,  $-P(O)CH_3-$ , and the like, as well as an appropriate substituted carbon-nitrogen double bond which links  $R^3$  and/or  $R^4$  to the hydrazino linkage.

As employed herein, "alkyl" refers to alkyl groups having in the range of 1 up to 8 carbon atoms;

20 "lower alkyl" refers to alkyl groups having in the range of 1 up to 4 carbon atoms; and "substituted alkyl" or "substituted lower alkyl" comprises alkyl (or lower alkyl) groups further bearing one or more substituents selected from halogen, cyano, nitro, hydroxy, alkoxy (-

25 OR), thioalkyl (-SR),  $-NR_2$ ,  $-NRC(O)R$ ,  $-OC(O)R$ ,  $-C(O)OR$ ,  $-C(O)NR_2$ ,  $-C(O)R$ , wherein each R is independently hydrogen or lower alkyl, and the like.

As employed herein, "cycloalkyl" refers to cyclic ring-containing groups containing in the range of

30 about 5 up to 8 carbon atoms, and "substituted cycloalkyl" refers to cycloalkyl groups further bearing one or more substituents as set forth above, as well as lower alkyl.

As employed herein, "heterocyclic" refers to cyclic (i.e., ring-containing) groups containing one or more (up to four) heteroatoms (e.g., N, O, S, or the like) as part of the ring structure, and having in the 5 range of 2 up to 5 nuclear carbon atoms and "substituted heterocyclic" refers to heterocyclic groups further bearing one or more substituents as set forth above, as well as lower alkyl.

As employed herein, "alkenyl" refers to 10 straight or branched chain hydrocarbyl groups having at least one carbon-carbon double bond, and having in the range of about 2 up to 12 carbon atoms, and "substituted alkenyl" refers to alkenyl groups further bearing one or more substituents as set forth above.

As employed herein, "alkynyl" refers to 15 straight or branched chain hydrocarbyl groups having at least one carbon-carbon triple bond, and having in the range of about 2 up to 12 carbon atoms, and "substituted alkynyl" refers to alkynyl groups further bearing one or 20 more substituents as set forth above.

As employed herein, "aryl" refers to aromatic groups having in the range of 6 up to 14 carbon atoms and "substituted aryl" refers to aryl groups further bearing one or more substituents as set forth above, as well as 25 lower alkyl.

As employed herein, "heteroaryl" refers to aromatic groups containing one or more heteroatoms (e.g., N, O, S, or the like) as part of the ring structure, and having in the range of 3 up to 14 carbon atoms and 30 "substituted heteroaryl" refers to heteroaryl groups further bearing one or more substituents as set forth above.

Presently preferred ecdysone mimics contemplated for use herein include compounds wherein R<sup>1</sup> is hydrogen; R<sup>2</sup> is an alkyl group possessing considerable bulk (such as, for example, alkyl groups containing a 5 tertiary carbon center, e.g., -C(R'')<sub>3</sub>, wherein each R'' is methyl or greater). Examples of alkyl groups having sufficient bulk for use herein include tert-butyl, sec-butyl, isopropyl, isobutyl, cyclohexyl, cyclopentyl, dicyclopropylmethyl, (cyclohexyl)ethyl, and the like); X<sup>1</sup> 10 and X<sup>2</sup> are both -C(O)-; R<sup>3</sup> is phenyl, substituted phenyl (with hydroxy, alkoxy, halo and/or substituted amino substituents being preferred, with 3,4-disubstitution pattern being especially preferred), heterocyclic (e.g., pyridyl or pyrimidine) or substituted heterocyclic (with 15 halo, alkyl, thioalkyl, hydroxy, alkoxy, and/or amino substituents being preferred); and R<sup>4</sup> is phenyl or substituted phenyl, heteroaryl or substituted heteroaryl or a bulky alkyl or cycloalkyl group.

Especially preferred ecdysone mimics 20 contemplated for use herein include N'-(3,5-dimethylbenzoyl)-N-(4-ethylbenzoyl)-N'-(tert-butyl) hydrazine, N,N'-dibenzoyl-N'-(tert-butyl) hydrazine, N'-(3,5-dimethylbenzoyl)-N-(4-ethylbenzyl)-N'-(tert-butyl) hydrazine, N'-(3,5-dimethylbenzoyl)-N-(2-methyl-25 3,4-(ethylenedioxy)-benzoyl)-N'-(tert-butyl) hydrazine, 3,5-di-tert-butyl-4-hydroxy-N-isobutyl-benzamide, 8-O-acetylharpagide, and the like.

Ecdysone or ecdysone-like ligands contemplated for use in the practice of the present invention are 30 characterized as not normally being present in the cells of the subject, meaning that the first ligand is exogenous to the subject. Ecdysteroids, for example, are not naturally present in mammalian systems. Thus, in accordance with the invention method, unless and until an

ecdysteroid is administered to the subject, substantially no expression of the desired gene occurs.

As readily recognized by those of skill in the art, it may be desirable to be able to rapidly induce or 5 rapidly turn off expression utilizing the regulatory system of the invention. This can readily be accomplished by administration of a suitable ecdysone antagonist before or after induction of the system (e.g., to prevent undesired activation of the system, to promote 10 rapid induction, to rapidly terminate expression, and the like). Numerous naturally-occurring and synthetic ecdysone antagonists are known in the art, e.g., ajugalactone.

The second ligand contemplated for use in the 15 practice of the present invention is a compound which interact (directly or indirectly) with a silent partner for the modified ecdysone receptor described herein. While such ligands alone impart virtually no activity to the invention expression system, they have been 20 discovered to greatly enhance the ability of ecdysone or ecdysone-like compounds to activate the invention expression system. Those of skill in the art can readily determine suitable ligands for the silent partner being employed. A presently preferred silent partner is RXR; 25 exemplary RXR agonists contemplated for use herein include 9-*cis*-retinoic acid, 4-(1-(3,5,5,8,8-pentamethyl-5,6,7,8-tetrahydro-2-naphthyl)-ethenyl) benzoic acid (3-methyl-TTNEB; LGD 1069), ((E)-2-(2-(5,6,7,8-tetrahydro-3,5,5,8,8-pentamethyl-2-naphthyl)propen-1-yl)-4-thiophenecarboxylic acid) (AGN 191701), 2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthyl)-2-(carboxyphenyl)-1,3-dioxolane (SR 11237), 4-(5H-2,3-(2,5-dimethyl-2,5-hexano)-5-methyl-dibenzo(b,e)(1,4)diazepin-11-yl)-benzoic acid (HX600) or 35 thiadiazepin analogs thereof, 3,7,11,15-tetramethyl

hexadecanoic acid (phytanic acid), 6-(1-(3,5,5,8,8-pentamethyl-5,6,7,8-tetrahydronaphthalen-2-yl)cyclopropyl)nicotinic acid (LG1000268), 2-(4-carboxyphenyl)-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1,3-dithiane (SR11203), 4-(2-methyl)-1-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)propenyl)benzoic acid (SR11217), and the like.

An effective amount of each of first and second ligand contemplated for use in the practice of the present invention is the amount of each ligand required to achieve the desired level of gene expression product. Ligand(s) can be administered in a variety of ways, as are well-known in the art. For example, such ligands can be administered topically, orally, intravenously, intraperitoneally, intravascularly, and the like. The first and second ligand can be administered concurrently or independently.

In accordance with a particular embodiment of the present invention, pharmaceutically acceptable formulations, and kits thereof, comprising at least one activator for a modified ecdysone receptor as described herein, at least one ligand for a silent partner for the modified ecdysone receptor, and a pharmaceutically acceptable carrier are contemplated. In accordance with another aspect of the present invention, pharmaceutically acceptable formulations consisting essentially of at least one activator for a modified ecdysone receptor as described herein, at least one ligand for a silent partner for the modified ecdysone receptor, and a pharmaceutically acceptable carrier, are contemplated. Pharmaceutical formulations of the present invention can be used in the form of a solid, a solution, an emulsion, a dispersion, a micelle, a liposome, and the like, wherein the resulting formulation contains one or more

activator(s) for a modified ecdysone receptor as described herein and one or more ligand(s) for a silent partner for the modified ecdysone receptor, as active ingredients, in admixture with an organic or inorganic 5 carrier or excipient suitable for enteral or parenteral applications.

The active ingredients may be compounded, for example, with the usual non-toxic, pharmaceutically acceptable carriers suitable for oral, topical, nasal, 10 transdermal, intravenous, subcutaneous, intramuscular, intracutaneous, intraperitoneally, intravascular and the like administration. Administration in the form of creams, lotions, tablets, dispersible powders, granules, syrups, elixirs, sterile aqueous or non-aqueous 15 solutions, suspensions or emulsions, and the like, is contemplated. Exemplary pharmaceutically acceptable carriers include carriers for tablets, pellets, capsules, suppositories, solutions, emulsions, suspensions, and any other form suitable for use. Such carriers which can be 20 used include glucose, lactose, gum acacia, gelatin, mannitol, starch paste, magnesium trisilicate, talc, corn starch, keratin, colloidal silica, potato starch, urea, medium chain length triglycerides, dextrans, and other carriers suitable for use in manufacturing preparations, 25 in solid, semisolid, or liquid form. In addition auxiliary, stabilizing, thickening and coloring agents and perfumes may be used. The active compound (i.e., ecdysteroid and/or retinoid as described herein) included in the pharmaceutically acceptable formulation in an 30 amount sufficient to produce the desired effect upon the process or condition of diseases.

Pharmaceutically acceptable formulations containing the active ingredients may be in a form suitable for oral use, for example, as aqueous or oily 35 suspensions, syrups or elixirs, tablets, troches,

lozenges, dispersible powders or granules, emulsions, or hard or soft capsules. For the preparation of oral liquids, suitable carriers include emulsions, solutions, suspensions, syrups, and the like, optionally containing 5 additives such as wetting agents, emulsifying and suspending agents, dispersing agents, sweetening, flavoring, coloring, preserving and perfuming agents, and the like. Formulations intended for oral use may be prepared according to any method known to the art for the 10 manufacture of pharmaceutically acceptable formulations.

Tablets containing the active ingredients in admixture with non-toxic pharmaceutically acceptable excipients may also be manufactured by known methods. The excipients used may be, for example, (1) inert 15 diluents such as calcium carbonate, lactose, calcium phosphate or sodium phosphate; (2) granulating and disintegrating agents such as corn starch, potato starch or alginic acid; (3) binding agents such as gum tragacanth, corn starch, gelatin or acacia, and 20 (4) lubricating agents such as magnesium stearate, stearic acid or talc. The tablets may be uncoated or they may be coated by known techniques to delay disintegration and absorption in the gastrointestinal tract and thereby provide a sustained action over a 25 longer period. For example, a time delay material such as glyceryl monostearate or glyceryl distearate may be employed. They may also be coated by the techniques described in the U.S. Pat. Nos. 4,256,108; 4,160,452; and 4,265,874, to form osmotic therapeutic tablets for 30 controlled release.

In some cases, formulations for oral use may be in the form of hard gelatin capsules wherein the active ingredients are mixed with an inert solid diluent, for example, calcium carbonate, calcium phosphate or kaolin. 35 They may also be in the form of soft gelatin capsules

wherein the active ingredient is mixed with water or an oil medium, for example, peanut oil, liquid paraffin, or olive oil.

Pharmaceutically acceptable formulations may be 5 in the form of a sterile injectable suspension. Suitable carriers include non-toxic parenterally-acceptable sterile aqueous or non-aqueous solutions, suspensions, or emulsions. Such a suspension may be formulated according to known methods using suitable dispersing or wetting 10 agents and suspending agents. They can also be manufactured in the form of sterile water, or some other sterile injectable medium immediately before use. Sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland 15 fixed oil may be employed including synthetic mono- or diglycerides, fatty acids (including oleic acid), naturally occurring vegetable oils like sesame oil, coconut oil, peanut oil, cottonseed oil, etc., or synthetic fatty vehicles like ethyl oleate or the like. 20 They may be sterilized, for example, by filtration through a bacteria-retaining filter, by incorporating sterilizing agents into the formulations, by irradiating the formulations, or by heating the formulations. Sterile injectable suspensions may also contain adjuvants 25 such as preserving, wetting, emulsifying, and dispersing agents. Buffers, preservatives, antioxidants, and the like can be incorporated as required.

Compounds contemplated for use in the practice of the present invention may also be administered in the 30 form of suppositories for rectal administration of the drug. These formulations may be prepared by mixing the drug with a suitable non-irritating excipient, such as cocoa butter, synthetic glyceride esters of polyethylene glycols, which are solid at ordinary temperatures, but

liquefy and/or dissolve in the rectal cavity to release the drug.

The pharmaceutically acceptable formulations are administered in a manner compatible with the route of 5 administration, the dosage formulation, and in a therapeutically effective amount. The required dosage will vary with the particular treatment desired, the degree and duration of therapeutic effect desired, the judgment of the practitioner, as well as properties 10 peculiar to each individual. Moreover, suitable dosage ranges for systemic application depend on the route of administration. It is anticipated that dosages between about 10 micrograms and about 10 milligram per kilogram of body weight per day will be used for therapeutic 15 treatment, with about 5 milligram per kilogram of body weight per day preferred.

An effective amount of the pharmaceutically acceptable formulation contemplated for use in the practice of the present invention is the amount of the 20 pharmaceutically acceptable formulation (i.e., first and second ligand) required to achieve the desired level of transcription and/or translation of exogenous nucleic acid. A therapeutically effective amount is typically an amount of each of the first and second ligands (or 25 precursor(s) therefor) that, when administered in a pharmaceutically acceptable formulation, is sufficient to achieve a plasma concentration of the transcribed or expressed nucleic acid product from about 0.1  $\mu$ g/ml to about 100  $\mu$ g/ml, preferably from about 1.0  $\mu$ g/ml to about 30 50  $\mu$ g/ml, more preferably at least about 2  $\mu$ g/ml and usually 5 to 10  $\mu$ g/ml.

Pharmaceutically acceptable formulations containing suitable ligand(s) are preferably administered intravenously, as by injection of a unit dose, for

example. The term "unit dose," when used in reference to a pharmaceutically acceptable formulation of the present invention, refers to a quantity of the pharmaceutical formulation suitable as unitary dosage for the subject, 5 each unit containing a predetermined quantity of active material(s) calculated to produce the desired therapeutic effect in association with the required diluent, i.e., carrier, or vehicle. It may be particularly advantageous to administer such formulations in depot or long-lasting 10 form as discussed hereinafter.

Suitable regimes for initial administration and booster shots are variable, but are typified by an initial administration followed by repeated doses at one or more intervals by a subsequent injection or other 15 administration. Alternatively, continuous intravenous infusion sufficient to maintain concentrations in the blood in the ranges specified for in vivo therapies are contemplated.

Response elements contemplated for use in the 20 practice of the present invention include elements recognized by the DNA-binding domain of the modified ecdysone receptor. In a preferred embodiment of the present invention, such elements are exogenous regulatory elements not normally present in the cells of the host or 25 are native response elements which have been engineered so as to no longer be capable of binding endogenous transcription factors. One class of exogenous regulatory elements contemplated for use herein includes hormone response elements which modulate transcription of 30 exogenous nucleic acid when bound to the DNA binding domain of the modified ecdysone receptor. Exemplary response elements include native and modified hormone response elements, GAL4 response elements, synthetic response elements (Pomerantz et al., *supra*), and the 35 like. In a preferred embodiment of the present

invention, modified ecdysone response elements are contemplated.

Ecdysone response elements contemplated for use in the practice of the present invention (relating to 5 modulation of the expression of exogenous genes in a subject) include native, as well as modified ecdysone response elements. Since invention modified ecdysone receptors can function as either homodimers or as heterodimers (with a silent partner therefor), any 10 response element that is responsive to an invention modified ecdysone receptor, in the form of a homodimer or heterodimer, is contemplated for use in the invention methods described herein. As is readily recognized by those of skill in the art, modified receptors according 15 to the invention (whether in the form of a homodimer or a heterodimer) can bind to either a response element having an inverted repeat motif (i.e., two or more half sites in mirror image orientation with respect to one another), or to a response element having a direct repeat motif.

20 In a preferred embodiment of the invention, invention modified ecdysone response elements are engineered so as to no longer be capable of binding to a farnesoid hormone receptor (since the mammalian farnesoid hormone receptor is able to bind to native ecdysone 25 receptor response element). Invention modified ecdysone response elements provide low background expression levels of the exogenous gene and increase the selectivity of the gene expression system when used in mammalian systems.

30 Ecdysone response elements contemplated for use herein are short cis-acting sequences (i.e., having about 12-20 bp) that are required for activation of transcription in response to an ecdysteroid, associated with a modified ecdysone receptor. The association of

these response elements with otherwise ecdysone-nonresponsive regulatory sequences causes such regulatory sequences to become ecdysone responsive. Ecdysone response element sequences function in a position- and 5 orientation-independent fashion.

The native ecdysone response element has been previously described, see, e.g., Yao et al., Cell, 71:63-72, 1992. Modified ecdysone response elements according to present invention comprise two half-sites (in either 10 direct repeat or inverted repeat orientation to one another), separated by a spacer of 0-5 nucleotides. As used herein, the term "half-site" refers to a contiguous 6 nucleotide sequence that is bound by a particular member of the steroid/thyroid hormone superfamily of 15 receptors. Each half-site is typically separated by a spacer of 0 up to about 5 nucleotides. Typically, two half-sites with a corresponding spacer make up a hormone response element. Hormone response elements can be incorporated in multiple copies into various 20 transcription regulatory regions.

Preferred modified ecdysone response elements according to the invention comprise, in any order, a first half-site and a second half-site separated by a spacer of 0-5 nucleotides;

25 wherein the first and second half-sites are inverted with respect to each other;

wherein said first half-site has the sequence:

-RGBNNM-,

30 (or complements thereof) wherein

each R is independently selected from A or G;

each B is independently selected from G, C, or T;

each N is independently selected from A, T, C, 35 or G; and

each M is independently selected from A or C, with the proviso that at least 4 nucleotides of each -RGBNNM- group of nucleotides are identical with the nucleotides at comparable positions of the sequence 5 -AGGTCA-; and

said second half-site is obtained from a glucocorticoid receptor subfamily response element.

The complement to the -RGBNNM- sequence set forth above is:

10 - YCVNNK-,

wherein

each Y is independently selected from T or C; each V is independently selected from C, G, or A;

15 each N is independently selected from A, T, C, or G; and

each K is independently selected from T or G.

Exemplary first half-sites having the -RGBNNM- motif for use in the invention modified ecdysone response 20 element include, for example, half-sites selected from -AGGGCA-, -AGTTCA-, -AGGTAA-, -AGGTCA-, -GGTTCA-, -GGGTTA-, -GGGTGA-, -AGGTGA-, or -GGGTCA-. A particularly preferred first half-site is -AGTGCA-.

Glucocorticoid receptor subfamily response 25 elements contemplated for use in the practice of the present invention are response elements having half-sites that are typically bound by glucocorticoid, mineralocorticoid, progesterone or androgen receptors. Suitable half-sites from glucocorticoid receptor 30 subfamily response elements can be selected from the following sequence (in either orientation):

-RGNNA-

(or complements thereof such as -YCNNGT-), wherein R, Y and N are as defined above. Exemplary half-sites having

the -RGNNA- motif for use in the invention modified ecdysone response element include -AGAAC-, -GGAACA-, -AGTTCA-, -AGGTCA-, -GGAACA-, -GGTTCA-, -GGGTCA-, -GGGTCA-, -AGGTGA-, -GGGTCA-, and the like, as well as 5 complements thereof. Particularly preferred half-sites having the -RGNNA- motif include -AGAAC- and -GGAACA-, with -AGAAC- being especially preferred.

When the above-described modified ecdysone response elements are employed to bind invention 10 heterodimeric receptors, the second half-site is inverted with respect to the first half-site. For example, when describing a single-strand of an invention modified ecdysone response element in the 5'-3' direction, the following general motif can be employed:

15 RGBNNM- (N)<sub>x</sub>-TGNNCY (SEQ ID NO:4),

where x is an integer of 0 up to about 5, with x = 1 being especially preferred. As an alternative orientation to the above described response element motif (SEQ ID NO:4), an invention response element can be 20 described in the 5'-3' direction as:

RGNNA- (N)<sub>x</sub>-KNNVCY (SEQ ID NO:5),

where x is an integer of 0 up to about 5, with x = 1 being especially preferred.

In preferred embodiments of the present 25 invention, the first half-site is obtained from an ecdysone response element and the second half-site is obtained from a hormone response element selected from a glucocorticoid response element, a mineralocorticoid response element, a progesterone response element or an 30 androgen response element. In a particularly preferred embodiment of the present invention, the first half-site

is obtained from an ecdysone response element and the second half-site is obtained from a glucocorticoid response element.

5 In a particularly preferred embodiment of the invention modified ecdysone response element, the first half-site is AGTGCA and the second half-site is TGTTCT. The presently most preferred modified-ecdysone response element for use in the invention methods is:

AGTGCA-N-TGTTCT (SEQ ID NO:6).

10 In another aspect of the invention, when modified ecdysone receptors of the invention exist as homodimers, response elements employed preferably have a direct repeat motif (instead of the above-described inverted repeat motif), as follows:

15 RGBNNM-(N)<sub>x'</sub>-RGBNNM (SEQ ID NO:7),

where R, B, N and M are as previously defined, and x' is an integer of 0 up to about 5, with x' = 3 being especially preferred.

20 Invention modified ecdysone response elements are characterized as having substantially no constitutive activity, which refers to the substantial absence of background levels of gene expression initiated by invention modified ecdysone response elements when introduced into mammalian expression systems. Since it 25 has been found that mammalian farnesoid hormone receptors are able to bind to and transactivate gene expression from native ecdysone response elements, in certain embodiments of the present invention (e.g., where it is desired to avoid farnesoid-mediated background expression), modified ecdysone response elements are employed (because such response elements typically have

substantially no binding affinity for farnesoid X receptor (FXR).

Presently preferred invention modified ecdysone response elements are further characterized as having 5 substantially no binding affinity for farnesoid X receptor (FXR), i.e., invention response elements are incapable of binding FXR (which would thereby create undesired background levels of expression). Thus, 10 presently preferred invention modified ecdysone response elements preferably induce basal levels of expression of substantially zero.

Response elements employed in the practice of the present invention are operably linked to a suitable promoter for expression of exogenous gene product(s). As 15 used herein, the term "promoter" refers to a specific nucleotide sequence recognized by RNA polymerase, the enzyme that initiates RNA synthesis. This sequence is the site at which transcription can be specifically initiated under proper conditions. When exogenous genes, 20 operatively linked to a suitable promoter and ecdysone response elements, are introduced into the cells of a suitable host, expression of the exogenous genes is controlled by the presence of ecdysteroid compounds, which are not normally present in the host cells.

25 In accordance with another embodiment of the present invention, there are provided methods of inducing the expression of an exogenous gene in a mammalian subject containing:

- 30 (i) a DNA construct comprising an exogenous gene operatively associated with a response element,
- (ii) DNA encoding a modified ecdysone receptor under the control of an

inducible promoter; wherein said modified ecdysone receptor, in the optional presence of a receptor capable of acting as a silent partner therefor, is capable of binding to said response element, and

5 (iii) a first ligand and a second ligand,

wherein said first ligand is:

10 a ligand for said modified ecdysone receptor, not normally present in the cells of said subject, and

not toxic to said subject; and

wherein said second ligand is a ligand for said silent partner;

15 said method comprising subjecting said subject to conditions suitable to induce expression of said modified ecdysone receptor.

Inducible promoters contemplated for use in the practice of the present invention are transcription 20 regulatory regions that do not function to transcribe mRNA unless inducing conditions are present. Examples of suitable inducible promoters include DNA sequences corresponding to: the E. coli lac operator responsive to IPTG (see Nakamura et al., Cell, 18:1109-1117, 1979); the 25 metallothionein promoter metal-regulatory-elements responsive to heavy-metal (e.g. zinc) induction (see Evans et. al, U.S. Patent No. 4,870,009), the phage T7lac promoter responsive to IPTG (see Studier et al., Meth. Enzymol., 185: 60-89, 1990; and U.S. Patent No. 30 4,952,496), the heat-shock promoter, and the like.

In accordance with another embodiment of the present invention, there are provided methods of inducing expression of an exogenous gene in a mammalian subject

containing a DNA construct comprising said exogenous gene under the control of a response element, said method comprising introducing into said subject:

5 a modified ecdysone receptor;  
a first ligand; and  
a second ligand,

wherein said first ligand is:

a ligand for said modified ecdysone receptor,  
not normally present in the cells of said  
10 subject, and

not toxic to said subject; and

wherein said second ligand is a ligand for said silent  
partner;

15 wherein said receptor, optionally in  
combination with a receptor capable of acting as a silent  
partner therefor, and ligand(s) therefor, binds to said  
response element, activating transcription therefrom.

20 In accordance with another embodiment of the  
present invention, there are provided methods for the  
expression of recombinant products detrimental to a host  
organism, said method comprising:

transforming suitable host cells with:

25 (i) a DNA construct encoding said  
recombinant product operatively  
associated with a response element,  
and

(ii) DNA encoding a modified ecdysone  
receptor, wherein said modified  
ecdysone receptor forms a homodimer,  
30 or a heterodimer with a silent  
partner therefor;

growing said host cells in suitable media; and

inducing expression of said recombinant product by introducing into said host cells a first ligand and a second ligand,

wherein said first ligand is:

5 a ligand for said modified ecdysone receptor, not normally present in the cells of said subject, and

not toxic to said subject; and

wherein said second ligand is a ligand for said silent 10 partner component of said heterodimer; and

optionally introducing into said host cells a receptor capable of acting as a silent partner for said modified ecdysone receptor.

Recombinant products detrimental to a host 15 organism contemplated for expression in accordance with the present invention include any gene product that functions to confer a toxic effect on the organism. For example, inducible expression of a toxin such as the diphtheroid toxin would allow for inducible tissue 20 specific ablation (Ross et al. (1993) Genes and Development 7, 1318-1324). Thus, the numerous gene products that are known to induce apoptosis in cells expressing such products are contemplated for use herein (see, e.g., Apoptosis, The Molecular Basis of Cell Death, 25 Current Communications In Cell & Molecular Biology, Cold Spring Harbor Laboratory Press, 1991).

Suitable media contemplated for use in the practice of the present invention include any growth and/or maintenance media, but exclude first and second 30 ligand as described above.

In accordance with another embodiment of the present invention, there are provided nucleic acids

encoding modified ecdysone receptors as described herein. Such nucleic acids can be incorporated into various expression vectors known to those of skill in the art. Preferred nucleic acids encode constructs described in 5 Figure 1D.

In accordance with another embodiment of the present invention, there are provided gene transfer vectors useful for the introduction of invention constructs into suitable host cells. Such gene transfer 10 vectors comprise a transcription regulatory region having a minimal promoter (i.e., a promoter region that does not have an enhancer), and an invention modified ecdysone response element, wherein said regulatory region is operatively associated with DNA containing an exogenous 15 gene, and wherein said modified ecdysone response element is present in multiple copies. The number of copies of response elements can readily be varied by those of skill in the art. For example, transcription regulatory regions can contain from 1 up to about 50 20 copies of a particular response element, preferably 2 up to about 25 copies, more preferably 3 up to about 10-15 copies, with about 4-6 copies being especially preferred.

Gene transfer vectors (also referred to as "expression vectors") contemplated for use herein are 25 recombinant nucleic acid molecules that are used to transport exogenous nucleic acid into cells for expression and/or replication thereof. Expression vectors may be either circular or linear, and are capable of incorporating a variety of nucleic acid constructs 30 therein. Expression vectors typically come in the form of a plasmid that, upon introduction into an appropriate host cell, results in expression of the inserted DNA.

As used herein, the phrase "transcription regulatory region" refers to the region of a gene or

expression construct that controls the initiation of mRNA transcription. Regulatory regions contemplated for use herein typically comprise at least a minimal promoter in combination with an ecdysone response element. A minimal 5 promoter, when combined with an enhancer region (e.g., a hormone response element), functions to initiate mRNA transcription in response to a ligand/receptor complex. However, transcription will not occur unless the required inducer (ligand) is present.

10 As used herein, the phrase "operatively associated with" refers to the functional relationship of DNA with regulatory and effector sequences of nucleotides, such as promoters, enhancers, transcriptional and translational stop sites, and other 15 signal sequences. For example, operative linkage of DNA to a promoter refers to the physical and functional relationship between the DNA and promoter such that the transcription of such DNA is initiated from the promoter by an RNA polymerase that specifically recognizes, binds 20 to and transcribes the DNA.

Preferably, the transcription regulatory region further comprises a binding site for at least one ubiquitous transcription factor. Such a binding site is preferably positioned between the promoter and modified 25 ecdysone response element of the invention. Suitable ubiquitous transcription factors for use herein are well-known in the art and include, for example, Sp1.

Expression vectors suitable for use in the practice of the present invention are well known to those 30 of skill in the art and include those that are replicable in eukaryotic cells and/or prokaryotic cells as well as those that remain episomal and those that integrate into the host cell genome. Expression vectors typically further contain other functionally important nucleic acid

sequences, such as expression constructs encoding antibiotic resistance proteins, and the like.

Exemplary eukaryotic expression vectors include eukaryotic constructs, such as the pSV-2 gpt system 5 (Mulligan et al., Nature, 1979, 277:108-114); pBlueScript (Stratagene, La Jolla, CA), the expression cloning vector described by Genetics Institute (Science, 1985, 228:810-10 815), and the like. Each of these plasmid vectors are capable of promoting expression of the invention chimeric protein of interest.

Promoters, depending upon the nature of the regulation, may be constitutively or inducibly regulated, or may be tissue-specific (e.g., expressed only in T-cells, endothelial cells, smooth muscle cells, and the 15 like). Exemplary promoters contemplated for use in the practice of the present invention include the SV40 early promoter, the cytomegalovirus (CMV) promoter, the mouse mammary tumor virus (MMTV) steroid-inducible promoter, Moloney murine leukemia virus (MMLV) promoter, elongation 20 factor 1 $\alpha$  (EF1 $\alpha$ ) promoter, albumin promoter, APO A1 promoter, cyclic AMP dependent kinase II (CaMKII) promoter, kevatin promoters, CD3 promoter, immunoglobulin light or heavy chain promoters, neurofilament promoter, neuron specific enolase promoter, L7 promoter, CD2 25 promoter, myosin light chain kinase promoter, HOX gene promoter, thymidine kinase (TK) promoter, RNA Pol II promoter, MYOD promoter, MYF5 promoter, phosphoglycerokinase (PGK) promoter, Stf1 promoter, Low Density Lipoprotein (LDL) promoter, and the like.

30 Suitable means for introducing (transducing) expression vectors containing invention nucleic acid constructs into host cells to produce transduced recombinant cells (i.e., cells containing recombinant heterologous nucleic acid) are well-known in the art

(see, for review, Friedmann, 1989, Science, 244:1275-1281; Mulligan, 1993, Science, 260:926-932, each of which are incorporated herein by reference in their entirety). Exemplary methods of transduction include, e.g., 5 infection employing viral vectors (see, e.g., U.S. Patent 4,405,712 and 4,650,764), calcium phosphate transfection (U.S. Patents 4,399,216 and 4,634,665), dextran sulfate transfection, electroporation, lipofection (see, e.g., U.S. Patents 4,394,448 and 4,619,794), cytofection, 10 particle bead bombardment, and the like. Heterologous nucleic acid(s) contained within expression vectors can optionally include sequences which allow for extrachromosomal (i.e., episomal) maintenance, or the heterologous nucleic acid can be donor nucleic acid that 15 integrates into the genome of the host.

In a specific embodiment, said expression transfer vector is a viral vector, preferably a retroviral vector. Retroviral vectors are gene transfer plasmids wherein the heterologous nucleic acid resides 20 between two retroviral LTRs. Retroviral vectors typically contain appropriate packaging signals that enable the retroviral vector, or RNA transcribed using the retroviral vector as a template, to be packaged into a viral virion in an appropriate packaging cell line 25 (see, e.g., U.S. Patent 4,650,764).

Suitable retroviral vectors for use herein are described, for example, in U.S. Patents 5,399,346 and 5,252,479; and in WIPO publications WO 92/07573, WO 90/06997, WO 89/05345, WO 92/05266 and WO 92/14829, 30 incorporated herein by reference, which provide a description of methods for efficiently introducing nucleic acids into human cells using such retroviral vectors. Other retroviral vectors include, for example, mouse mammary tumor virus vectors (e.g., Shackleford et

al., 1988, PNAS, USA, 85:9655-9659), lentiviruses, and the like.

Various procedures are also well-known in the art for providing helper cells which produce retroviral vector particles which are essentially free of replicating virus. See, for example, U.S. Patent 4,650,764; Miller, Human Gene Therapy, 1:5-14 (1990); Markowitz, et al., Journal of Virology, 61(4):1120-1124 (1988); Watanabe, et al., Molecular and Cellular Biology, 3(12):2241-2249 (1983); Danos, et al., Proc. Natl. Acad. Sci., 85:6460-6464 (1988); and Bosselman, et al., Molecular and Cellular Biology, 7(5):1797-1806 (1987), which disclose procedures for producing viral vectors and helper cells which minimize the chances for producing a viral vector which includes a replicating virus.

Recombinant retroviruses suitable for carrying out the invention methods are produced employing well-known methods for producing retroviral virions. See, for example, U.S. Patent 4,650,764; Miller, Human Gene Therapy, 1:5-14 (1990); Markowitz, et al., Journal of Virology, 61(4):1120-1124 (1988); Watanabe, et al., Molecular and Cellular Biology, 3(12):2241-2249 (1983); Danos, et al., Proc. Natl. Acad. Sci., 85:6460-6464 (1988); and Bosselman, et al., Molecular and Cellular Biology, 7(5):1797-1806 (1987).

In accordance with another embodiment of the present invention, there are provided recombinant cells containing nucleic acid encoding modified ecdysone receptors as described herein optionally further containing expression constructs. Exemplary eukaryotic cells suitable for introducing invention expression vectors include, e.g., CV-1 cells, P19 cells and NT2/D1 cells (which are derived from human embryo carcinomas), ES cells (embryonic stem cells), COS cells, mouse L

cells, Chinese hamster ovary (CHO) cells, primary human fibroblast cells, human embryonic kidney cells, African green monkey cells, HEK 293 (ATCC accession #CRL 1573; U.S. Patent No. 5,024,939), Ltk<sup>-</sup> cells (ATCC accession 5 #CCL1.3), COS-7 cells (ATCC under accession #CRL 1651), DG44 cells (dhfr<sup>-</sup> CHO cells; see, e.g., Urlaub et al. (1986) Cell. Molec. Genet. 12: 555), cultured primary tissues, cultured tumor cells, and the like. Presently preferred cells include CV-1 and 293 cells.

10 In accordance with another embodiment of the present invention, there is provided a transgenic animal containing a nucleic acid encoding an invention modified ecdysone receptor. As used herein, the phrase "transgenic animal" refers to an animal that contains one 15 or more inheritable expression constructs containing a recombinant modified ecdysone receptor transgene and/or an exogenous gene under the transcription control of an invention modified ecdysone response element. Preferably, an invention transgenic animal also contains 20 one or more inheritable expression constructs containing a member of the steroid/thyroid hormone superfamily of receptors that functions as a silent partner for modified ecdysone receptor (e.g., RXR).

Methods of making transgenic animals using a 25 particular nucleic acid construct are well-known in the art. When preparing invention transgenic animals, it is preferred that two transgenic lines are generated. The first line will express, for example, RXR and a modified EcR (e.g., VpEcR). Tissue specificity is conferred by 30 the selection of tissue-specific promoters (e.g., T-cell specific) that will then direct the expression of the receptors. A second line contains an ecdysone responsive promoter controlling the expression of an exogenous cDNA.

In a preferred embodiment of the present invention, an invention transgenic animal contains one or more expression constructs containing nucleic acid encoding a modified ecdysone receptor, and an exogenous 5 gene under the transcription control of an invention modified ecdysone response element, and optionally, exogenous RXR. It has been found that in transgenic mice containing an ecdysone inducible promoter (i.e., an invention modified ecdysone response element) and 10 expressing a modified ecdysone receptor and RXR, treatment with a first and a second ligand (as described herein) can activate gene expression. Thus, with tissue specific expression of the modified ecdysone receptor and RXR and timely ligand addition, inducible gene expression 15 can be achieved with spatial, dosage, and temporal specificity.

In accordance with another embodiment of the present invention, there are provided methods for inducing expression of an exogenous gene in a transgenic 20 animal containing a modified ecdysone receptor as described herein, said method comprising:

introducing into said animal a DNA construct encoding an exogenous gene under the transcription control of a response element 25 responsive to said modified ecdysone receptor; and

administering to said animal an amount of a first ligand and a second ligand (as described herein) effective to induce 30 expression of said exogenous gene.

As discussed hereinbefore, the modified ecdysone receptor forms a homodimer, or optionally a heterodimer in the presence of a silent partner of the steroid/thyroid hormone superfamily of receptors, and functions to 35 activate transcription from an expression vector having a

response element responsive to the particular homodimer or heterodimer formed.

In accordance with another embodiment of the present invention, there are provided methods for the 5 induction of two different genes in a mammalian subject comprising: activating a first exogenous gene employing the invention ecdysone inducible system; and activating a second gene using an inducible gene expression system. Examples of inducible gene expression systems include 10 gene expression induced by tetracycline (i.e., tetracycline-inducible gene expression), mammalian steroid hormones or synthetic steroids thereof (i.e., RU-486-inducible gene expression), chemical inducer of dimerization (e.g., FK506, FK1012, CsA, rapamycin, FKCsA, 15 and the like; see, e.g., Rivera et al., Nat Med 2:1028-1032, 1996), and the like. The invention method for inducing two different genes is particularly advantageous because it permits the temporal, spatial, and dosage specific control of two exogenous genes.

20 The tetracycline inducible system is well-known in the art (see, e.g., Gossen et al. (1992) Proc. Natl. Acad. Sci. 89, 5547-5551; Gossen et al. (1993) TIBS 18, 471-475; Furth et al. (1994) Proc. Natl. Acad. Sci. 91, 9302-9306; and Shockett et al. (1995) Proc. Natl. Acad. Sci. 92, 6522-6526).

30 All U.S. and Foreign Patent publications, textbooks, and journal publications referred to herein are hereby expressly incorporated by reference in their entirety. The invention will now be described in greater detail by reference to the following non-limiting examples.

Example 1  
Preparation of modified ecdysone receptors

Plasmid preparation:

The plasmids CMX-EcR, CMX-USP, CMX-FXR,  
5 CMX-hRXRa, EcREx5-ΔMTV-Luc, CMX-GEcR, MMTV-luc, and  
CMX-GR have been previously described (Yao, et al.,  
Nature 366:476-479 (1993) and Forman, et al. Cell 81:687-  
693 (1995)).

The plasmid CMX-VpEcR was constructed by  
10 ligation of an EcoRI fragment of psk-EcR and CMX-Vp16.

The plasmid CMX-VgEcR was generated by  
site-directed mutagenesis of CMX-VpEcR using the  
Transformer Mutagenesis Kit (Clontech) and the mutagenic  
Oligonucleotide (SEQ ID NO:8) :

15 5'-TACAACGCCCTCACCTGTGGATCCTGCAAGGTGTTCTTCGACGCAGC-3'.

Mutagenesis of VpEcR to VgEcR altered the P-box region of  
the DNA binding domain of ecdysone receptor to resemble  
that of GR (Umesono and Evans, Cell 57:1139-1146 (1989)).  
The following amino acids in the DNA-binding domain of  
20 the ecdysone receptor were altered: E282G, G283S, and  
G286V (E=glutamate, G=glycine, S=serine, V=valine).

The reporter construct EcREx4-ΔHSP-β-gal was  
constructed by oligomerizing two annealed  
oligonucleotides containing the HSP-EcRE (Yao, et al.,  
25 Nature 366:476-479 (1993)).

EcREx4-Sp1x3-ΔHSP-βgal was constructed by  
ligating the following annealed oligonucleotides into the  
Asp718 site of EcREx4-HSP-β-gal (SEQ ID NO:9):

5' -GTA CCCC GGGG CGGG CTAT GCG GGG CGGG CTA ATCG CTAG GGG CGGG CA-  
3'

and (SEQ ID NO:10) :

5' -GTA CTG CCCC GCCC CTAG CGATT AG CCCC GCCC GCAT AG CCCC GCC  
5 CGGG A-3' .

ΔHSP is a minimal promoter derived from the Drosophila heat shock promoter with its enhancers deleted.

To generate the construct E/GRE<sub>4</sub>-ΔMTV-Luc, the following oligonucleotides (SEQ ID NO:11) :

10 5' -AGCTCGATGGACAAGTGCATTGTTCTTGCTGAA-3' ;

and (SEQ ID NO:12) :

5' -AGCTTTCAGCAAGAGAACAAATGCAC TTGTCCATCG-3' ,

were annealed, multimerized, and ligated into the HindIII site of ΔMTV-Luc. The resulting reporter contained 4  
15 copies of the invention modified ecdysone response element E/GRE.

To produce the plasmid pRC-ESH<sub>3</sub>, a BglII/(XhoI) fragment containing EcRE<sub>4</sub>-Splx3-ΔHSP-β-gal was subcloned into BglII/(NotI) digested pRC-CMV (Invitrogen, San  
20 Diego, CA), which contains a neomycin resistance gene.

Cell Culture and Transient Transfections:

CV-1 cells were maintained in DMEM supplemented with 10% Fetal Bovine Serum. Transient transfections were performed using DOTAP transfection reagent  
25 (Boehringer-Mannheim). Transfections using β-galactosidase as the reporter were assayed either by Galactolight luminescent assay (Tropix, Bedford, MA) or

by standard liquid ONPG assay (Sigma, St. Louis, MO). The values were normalized by co-transfection of CMX-luciferase. Transfections using luciferase as the reporter were assayed by standard techniques using 5 luciferin and ATP. These values were normalized by co-transfection of CMX- $\beta$ -galactosidase. Hormone treated cells were treated with ethanol, 50  $\mu$ M Juvenile Hormone III (Sigma), 1 $\mu$ M muristerone A (Zambon, Bresso, IT), or 1 $\mu$ M dexamethasone (Sigma) unless otherwise noted.

10 To maximize the sensitivity of the invention ecdysone inducible system, modifications of the ecdysone receptor were made. The N-terminal transactivation domain of the ecdysone receptor was replaced by the corresponding domain of the glucocorticoid receptor (GR), 15 resulting in the modified ecdysone receptor GEcR (See Figure 1D). CV-1 cells were transfected with the plasmid CMX-GEcR encoding the modified ecdysone receptor as discussed above. After transfection, cells were either treated with ethanol or 1 $\mu$ M muristerone A. This hybrid 20 modified ecdysone receptor boosted muristerone responsiveness from 3- to 11-fold in a transient transfection assay (Fig. 1A). Replacement of the natural heterodimeric partner for the ecdysone receptor, USP, by its mammalian homologue, the retinoid X receptor (RXR), 25 produced a more potent ligand dependent heterodimer, providing a 34 fold induction (Fig. 1A).

30 A more potent heterodimer, however, was obtained by combining RXR and VpEcR, an N-terminal truncation of the ecdysone receptor attached to the VP16 activation domain, resulting in a 212 fold induction (Fig. 1A and 1D). Different from most nuclear receptor/VP16 fusion proteins which exhibit high constitutive activity, VpEcR generates ligand dependent superinduction while maintaining a very low basal

activity (Underhill et al., Mol. Encod. 8:274-285 (1994) and Perlmann et al., Genes & Devel. 7:1411-1422 (1993)).

In addition, the reporter vector was also modified by inserting consensus binding sites for the 5 ubiquitous transcription factor Spl between the minimal promoter and the ecdysone response elements (Kamine et al., Proc. Natl. Acad. Sci. 88:8510-8514 (1991) and Strahle et al., EMBO 7:3389-3395 (1988)). The addition of Spl sites to the ecdysone responsive promoter 10 increases the absolute activity 5-fold (Fig. 1A).

Example 2Construction of a novel ecdysone response element

Although no mammalian transcription factors have been shown to have a natural enhancer element like 5 the ecdysone response element, which is composed of two inverted half-sites of the sequence AGGTCA spaced by one nucleotide, it is difficult to preclude such a possibility. The recently cloned farnesoid X receptor (FXR) can very weakly activate certain synthetic 10 promoters containing an ecdysone response element in response to extremely high concentrations of farnesoids (Forman et al., Cell 81:687-693 (1995)).

In FXR containing cells and in transgenic mice, activation of gene expression by endogenous receptors 15 would create undesirable background levels of reporter protein. To circumvent this potential problem, the DNA binding specificity of VpEcR was altered to mimic that of GR, which binds as a homodimer to an inverted repeat of the sequence AGAACCA, spaced by three nucleotides. This 20 altered binding specificity was achieved by mutating three amino acid residues of VpEcR in the P-box of the DNA binding domain, a region previously shown to be essential for DNA sequence recognition (Umesono and Evans, Cell 57:1139-1146 (1989)). This new hybrid 25 modified ecdysone receptor is referred herein as VgEcR and is responsive to a new hybrid response element referred to herein as the E/GRE (SEQ ID NO:6), which contains two different half-site motifs, RGBNNM and RGNNCNA, spaced by one nucleotide (Fig. 1B). This new 30 response element is a hybrid between the glucocorticoid response element (GRE) and that of type II nuclear receptors like RXR, EcR, retinoic acid receptor (RAR), thyroid hormone receptor (T3R), etc. Although FXR can activate a promoter containing the wild type ecdysone 35 response element, it cannot activate one containing the

E/GRE (Fig 1B; note log scale). The E/GRE reporter is not activated by GR nor does VgEcR activate a dexamethasone responsive promoter (Fig 1C).

Example 3

5 Assay for inducer effectiveness on the ecdysone-inducible expression system

CV-1 cells were maintained in DMEM supplemented with 10% fetal bovine serum. Transfections were performed using the Dotap transfection reagent 10 (Boehringer Mannheim). All candidate inducers were dissolved in either ethanol or DMSO. Muristerone A (MurA) was used as a positive control for activation. All transfections were performed in triplicate.

Subconfluent CV-1 cells were transiently 15 transfected with plasmids encoding the basic components of the invention ecdysone-inducible system (e.g., the VgEcR/RXR heterodimer) under the control of a strong constitutive promoter (e.g., CMX). The E/GRE<sub>4</sub>-ΔMTV-Luc plasmid is routinely used as a reporter. This plasmid 20 (see No et al., in Proc. Natl. Acad. Sci. USA 93:3346 (1996)) contains the firefly luciferase gene under the control of a minimal promoter and four binding sites for the VgEcR/RXR heterodimer. A CMX-β-gal reporter plasmid was included in the transfection as a control for 25 transfection efficiency. Approximately 10 hours after transfection, the media was removed and replaced with fresh media containing varying concentrations of the putative inducers. Cells were exposed to candidate activators for 20-48 hours, then assayed for luciferase 30 and β-galactosidase activity employing standard techniques. Luciferase values were normalized using β-galactosidase activity levels.

Figure 3 presents the results of testing with muristerone A alone over several concentrations, an RXR ligand (6-(1-(3,5,5,8,8-pentamethyl-5,6,7,8-tetrahydronaphthalen-2-yl)cyclopropyl)nicotinic acid (LG1000268)) alone over several concentrations, or a combination of the two, where the concentration of MurA varies while the concentration of LG1000268 remains constant (100nM).

10 The results presented in Figure 3 illustrate that RXR ligand alone has substantially no effect on this system, that muristerone alone is an effective inducer of the system, and that the combination of muristerone and RXR ligand dramatically enhances the activation of the system.

15

Example 4Assays with inducers other than muristerone

10 The benefits of combining ecdysone inducers with RXR agonists were further demonstrated employing other ecdysone agonists, besides muristerone. Thus, the procedure described in Example 3 was repeated with two different diacylhydrazines, i.e., N'-(3,5-dimethylbenzoyl)-N-(2-methyl-3,4-(ethylenedioxy)-benzoyl)-N'-(tert-butyl) hydrazine and N'-(3,5-dimethylbenzoyl)-N-(4-ethylbenzoyl)-N'-(tert-butyl) hydrazine (Figure 4). The enhanced ability of these compounds to activate the invention expression system in the further presence of an RXR agonist (e.g., (6-(1-(3,5,5,8,8-pentamethyl-5,6,7,8-tetrahydronaphthalen-2-yl)cyclopropyl)nicotinic acid (LG1000268)) is readily apparent upon inspection of the data presented in Figure 4.

Example 5Effect of various RXR ligands on induction activity  
of ponasterone A

The benefits of combining ecdysone inducers  
5 with RXR agonists were further demonstrated employing the  
ecdysone analog, ponasterone A (ponA), in combination  
with several different RXR agonists. Thus, the procedure  
described in Example 3 was repeated with ponasterone A  
and three different RXR agonists, i.e., LG1000268  
10 ((6-(1-(3,5,5,8,8-pentamethyl-5,6,7,8-  
tetrahydronaphthalen-2-yl)cyclopropyl)nicotinic acid),  
LGD 1069 (4-(1-(3,5,5,8,8-pentamethyl-5,6,7,8-  
tetrahydro-2-naphthyl)-ethenyl) benzoic acid) and 9-cis-  
15 RA (9-cis-retinoic acid). The enhanced ability of each  
of these RXR agonists to activate the invention  
expression system in combination with ponasterone A, is  
readily apparent upon inspection of the data presented in  
Figure 5.

While the invention has been described in  
20 detail with reference to certain preferred embodiments  
thereof, it will be understood that modifications and  
variations are within the spirit and scope of that which  
is described and claimed.

That which is claimed is:

1. A method for modulating the expression of an exogenous gene in a mammalian subject containing:

(i) a DNA construct comprising said exogenous gene is operatively associated with a response element; and

5

(ii) a modified ecdysone receptor which, in the optional presence of a silent partner therefor, is capable of binding to said response element;

10 said method comprising administering to said subject an effective amount of a first ligand and a second ligand,

wherein said first ligand is:

15 a ligand for said modified ecdysone receptor, not normally present in the cells of said subject, and

not toxic to said subject; and

wherein said second ligand is a ligand for said silent partner.

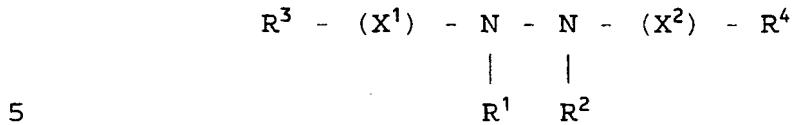
2. A method according to claim 1 wherein said first ligand is an ecdysteroid selected from the group consisting of a naturally occurring ecdysone, an ecdysone-analog and an ecdysone-mimic.

3. A method according to claim 2 wherein said naturally occurring ecdysone is  $\alpha$ -ecdysone or  $\beta$ -ecdysone.

4. A method according to claim 2 wherein said ecdysone-analog is ponasterone A, ponasterone B, ponasterone C, ponasterone D, 26-iodoponasterone A, muristerone A, inokosterone, 26-mesylinokosterone, 5 sidasterone, buterosterone, ajugasterone, makisterone, cyasterone or sengosterone.

5. A method according to claim 2 wherein said ecdysone-mimic is a 1,2-diacyl hydrazine, an N'-substituted-N,N'-disubstituted hydrazine, a dibenzoylalkyl cyanohydrazone, an N-substituted-N-alkyl-5 N,N-diaroyl hydrazine, an N-substituted-N-acyl-N-alkyl-carbonyl hydrazine, an N-aroyl-N'-alkyl-N'-aroyl hydrazine, a 3,5-dialkyl-4-hydroxy-N-alkyl-benzamide, or an 8-O-acetylharpagide.

6. A method according to claim 2 wherein said ecdysone-mimic has the formula:



5 wherein:

10 R<sup>1</sup> is optionally hydrogen, lower alkyl or substituted lower alkyl, alkenyl or substituted alkenyl, alkynyl or substituted alkynyl, aryl or substituted aryl, or heteroaryl or substituted heteroaryl, with the proviso that R<sup>1</sup> is not present when X<sup>1</sup> is part of a carbon-nitrogen double bond linking R<sup>3</sup> to the hydrazino group;

15 R<sup>2</sup> is optionally hydrogen, alkyl or substituted alkyl, or cyclohexyl or substituted cyclohexyl, with the proviso that R<sup>2</sup> is not present when X<sup>2</sup> is part of a carbon-nitrogen double bond linking R<sup>4</sup> to the hydrazino group;

$R^3$  and  $R^4$  are independently part of an  
appropriately substituted carbon-nitrogen double bond  
20 which links  $R^3$  and/or  $R^4$  to the hydrazino linkage, or  $R^3$   
and  $R^4$  are independently aryl or substituted aryl,  
heteroaryl or substituted heteroaryl, provided, however,  
that when two adjacent positions on the aryl or  
heteroaryl moieties are substituted with alkoxy,  
25 thioalkyl, alkylamino, or dialkylamino groups, these  
groups may be joined to form a 5- or 6- membered  
heterocyclic ring system, or  $R^3$  and  $R^4$  are independently  
heterocyclic or substituted heterocyclic, or cycloalkyl  
or substituted cycloalkyl; and

30  $X^1$  and  $X^2$  are independently  $-C(O)-$ ,  $-C(S)-$ ,  
 $-C(NR_2)-$ ,  $-C(=CN)NH-$ ,  $-C(O)O-$ ,  $-C(O)NH-$ ,  $-C(O)NHSO_2-$ ,  
 $-CH_2-$ ,  $-SO_2-$ ,  $-P(O)CH_3-$ , or an appropriate substituted  
carbon-nitrogen double bond which links  $R^3$  and/or  $R^4$  to  
the hydrazino linkage.

7. A method according to claim 2 wherein said  
ecdysone-mimic is  $N'-(3,5\text{-dimethylbenzoyl})-N-(4\text{-ethylbenzoyl})-N'-(tert-butyl)$  hydrazine,  $N,N'$ -dibenzoyl-  
 $N'-(tert-butyl)$  hydrazine,  $N'-(3,5\text{-dimethylbenzoyl})-N-(4\text{-ethylbenzoyl})-N'-(tert-butyl)$  hydrazine,  $N'-(3,5\text{-dimethylbenzoyl})-N-(2\text{-methyl-3,4-(ethylenedioxy)-benzoyl})-N'-(tert-butyl)$  hydrazine, 3,5-di-tert-butyl-4-hydroxy-N-isobutyl-benzamide or 8-O-acetylharpagide.

8. A method according to claim 1 wherein said receptor capable of acting as a silent partner is RXR.

9. A method according to claim 8 wherein said RXR is exogenous to said mammalian subject.

10. A method according to claim 8 wherein said second ligand is an RXR agonist.

11. A method according to claim 8 wherein said RXR agonist is 9-cis-retinoic acid, 4-(1-(3,5,5,8,8-pentamethyl-5,6,7,8-tetrahydro-2-naphthyl)-ethenyl)benzoic acid, ((E)-2-(2-(5,6,7,8-tetrahydro-5,5,8,8-pentamethyl-2-naphthyl)propen-1-yl)-4-thiophenecarboxylic acid, 2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthyl)-2-(carboxyphenyl)-1,3-dioxolane, 4-(5H-2,3-(2,5-dimethyl-2,5-hexano)-5-methyl-dibenzo(b,e)(1,4)diazepin-11-yl)-benzoic acid or 10 thiadiazepin analogs thereof, 3,7,11,15-tetramethyl hexadecanoic acid (phytanic acid), 6-(1-(3,5,5,8,8-pentamethyl-5,6,7,8-tetrahydronaphthalen-2-yl)cyclopropyl)nicotinic acid, 2-(4-carboxyphenyl)-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1,3-dithiane or 15 4-(2-methyl)-1-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)propenyl)benzoic acid.

12. A method according to claim 1 wherein said method further comprises administering to said subject an effective amount of an antagonist.

13. A method according to claim 12, wherein said antagonist is ajugalactone.

14. A method according to claim 1 wherein said exogenous gene is a wild type gene and/or therapeutic gene.

15. A method according to claim 14 wherein said wild type gene is selected from genes which encode products:

the substantial absence of which leads to 5 the occurrence of a non-normal state in said subject; or

a substantial excess of which leads to the occurrence of a non-normal state in said subject.

16. A method according to claim 14 wherein said therapeutic gene is selected from those which encode products:

5 which are toxic to the cells in which they are expressed; or

which impart a beneficial property to said subject.

17. A method according to claim 1 wherein said modified ecdysone receptor comprises:

5 a ligand binding domain capable of binding an ecdysteroid;

a DNA-binding domain; and

an activation domain,

wherein at least one of said DNA-binding domain or said activation domain is not obtained from a native ecdysone receptor.

18. A method according to claim 17, wherein said DNA binding domain is obtained from a DNA binding protein.

19. A method according to claim 17 wherein said modified ecdysone receptor is further characterized as having substantially no constitutive activity in mammalian cells.

20. A method according to claim 17 wherein the DNA-binding domain of said modified ecdysone receptor is derived from a member of the steroid/thyroid hormone superfamily of receptors.

21. A method according to claim 17 wherein said activation domain is obtained from a member of the steroid/thyroid hormone superfamily of receptors.

22. A method according to claim 17 wherein said activation domain is selected from a glucocorticoid receptor activation domain, a VP16 activation domain, a NF- $\kappa$ B activation domain or a GAL4 activation domain.

23. A method according to claim 22 wherein said modified ecdysone receptor is selected from VpEcR, VgEcR, Vp16-EcR-B1 or VgEcR-B1.

24. A method according to claim 23 wherein said modified ecdysone receptor is VgEcR.

25. A method according to claim 1 wherein said response element comprises, in any order, a first half-site and a second half-site separated by a spacer of 0-5 nucleotides;

5 wherein said first half-site has the sequence:

-RGBNNM-,

wherein

10 each R is independently selected from A or G; each B is independently selected from G, C, or T;

each N is independently selected from A, T, C, or G; and

15 each M is independently selected from A or C; with the proviso that at least 4 nucleotides of each -RGBNNM- group of nucleotides are identical with the nucleotides at comparable positions of the sequence -AGGTCA-; and

20 said second half-site is obtained from a glucocorticoid receptor subfamily response element.

26. A method according to claim 25 wherein said response element has substantially no binding affinity for farnesoid X receptor (FXR).

27. A method of inducing the expression of an exogenous gene in a mammalian subject containing:

- 5 (i) a DNA construct comprising an exogenous gene operatively associated with a response element,
- 10 (ii) DNA encoding a modified ecdysone receptor under the control of an inducible promoter; wherein said modified ecdysone receptor, in the optional presence of a receptor capable of acting as a silent partner therefor, is capable of binding said response element, and
- (iii) a first ligand and a second ligand,

15 wherein said first ligand is:

a ligand for said modified ecdysone receptor, not normally present in the cells of said subject, and  
not toxic to said subject; and

20 wherein said second ligand is a ligand for said silent partner,

said method comprising subjecting said subject to conditions suitable to induce expression of said modified ecdysone receptor.

28. A method of inducing expression of an exogenous gene in a mammalian subject containing a DNA construct containing said exogenous gene operatively associated with a response element, said method

5 comprising introducing into said subject:

a modified ecdysone receptor;  
a first ligand; and  
a second ligand;

wherein said first ligand is:

10           a ligand for said modified ecdysone receptor,  
         not normally present in the cells of said  
         subject, and  
         not toxic to said subject; and  
         wherein said second ligand is a ligand for said silent  
15           partner,

         wherein said receptor, in combination with a  
         receptor capable of acting as a silent partner therefor,  
         is capable of binding said response element, activating  
         transcription therefrom in the presence of suitable  
20           ligands.

29. A method for the expression of a  
recombinant product detrimental to a host organism, said  
method comprising:

transforming suitable host cells with:

5           (i) a DNA construct encoding said  
         recombinant product operatively  
         associated with a response element,  
         and  
         (ii) DNA encoding a modified ecdysone  
10           receptor, wherein said modified  
         ecdysone receptor is capable of  
         forming a heterodimer with a silent  
         partner therefor;

         growing said host cells in suitable media; and

15           inducing expression of said recombinant product  
by introducing into said host cells a first ligand and a  
second ligand,

wherein said first ligand is:

a ligand for said modified ecdysone receptor,

20 not normally present in the cells of said subject, and not toxic to said subject; and wherein said second ligand is a ligand for said silent partner component of said heterodimer; and

25 optionally introducing into said host cells a receptor capable of acting as a silent partner for said modified ecdysone receptor.

30. A pharmaceutically acceptable formulation comprising:

at least one activator for a modified ecdysone receptor, wherein said modified ecdysone receptor

5 comprises:

a ligand binding domain capable of binding an ecdysteroid;

a DNA-binding domain; and

an activation domain,

10 wherein at least one of said DNA-binding domain or said activation domain is not obtained from a native ecdysone receptor, and wherein said modified ecdysone receptor forms a heterodimer with a silent partner

15 therefor,

at least one activator for said silent partner for said modified ecdysone receptor, and

a pharmaceutically acceptable carrier.

31. A formulation according to claim 30 wherein said pharmaceutically acceptable carrier renders said formulation suitable for oral, topical, nasal, transdermal, intravenous, subcutaneous, intramuscular,

5 intracutaneous, intraperitoneal or intravascular administration.

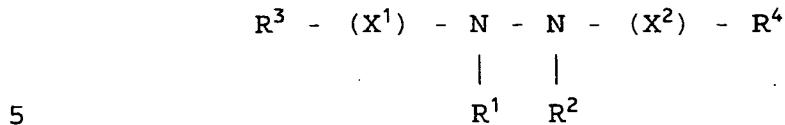
32. A formulation according to claim 30 wherein said ecdysteroid is a naturally occurring ecdysone, an ecdysone-analog or an ecdysone-mimic.

33. A formulation according to claim 32 wherein said naturally occurring ecdysone is  $\alpha$ -ecdysone or  $\beta$ -ecdysone.

34. A formulation according to claim 32 wherein said ecdysone analog is ponasterone A, ponasterone B, ponasterone C, ponasterone D, 26-iodoponasterone A, muristerone A, inokosterone, 5 26-mesylinokosterone, sidasterone, buterosterone, ajugasterone, makisterone, cyasterone or sengosterone.

35. A formulation according to claim 32 wherein said ecdysone-mimic is a 1,2-diacyl hydrazine, an N'-substituted-N,N'-disubstituted hydrazine, a dibenzoylalkyl cyanohydrazone, an N-substituted-N-alkyl-5 N,N-diaroyl hydrazine, an N-substituted-N-acyl-N-alkyl-carbonyl hydrazine, an N-arooyl-N'-alkyl-N'-arooyl hydrazine, a 3,5-dialkyl-4-hydroxy-N-alkyl-benzamide, or an 8-O-acetylharpagide.

36. A formulation according to claim 32 wherein said ecdysone-mimic has the formula:



wherein:

R<sup>1</sup> is optionally hydrogen, lower alkyl or substituted lower alkyl, alkenyl or substituted alkenyl, alkynyl or substituted alkynyl, aryl or substituted aryl, 10 or heteroaryl or substituted heteroaryl, with the proviso that R<sup>1</sup> is not present when X<sup>1</sup> is part of a carbon-nitrogen double bond linking R<sup>3</sup> to the hydrazino group;

R<sup>2</sup> is optionally hydrogen, alkyl or substituted alkyl, or cyclohexyl or substituted cyclohexyl, with the 15 proviso that R<sup>2</sup> is not present when X<sup>2</sup> is part of a carbon-nitrogen double bond linking R<sup>4</sup> to the hydrazino group;

R<sup>3</sup> and R<sup>4</sup> are independently part of an appropriately substituted carbon-nitrogen double bond 20 which links R<sup>3</sup> and/or R<sup>4</sup> to the hydrazino linkage, or R<sup>3</sup> and R<sup>4</sup> are independently aryl or substituted aryl, heteroaryl or substituted heteroaryl, provided, however, that when two adjacent positions on the aryl or heteroaryl moieties are substituted with alkoxy, 25 thioalkyl, alkylamino, or dialkylamino groups, these groups may be joined to form a 5- or 6- membered heterocyclic ring system, or R<sup>3</sup> and R<sup>4</sup> are independently heterocyclic or substituted heterocyclic, or cycloalkyl or substituted cycloalkyl; and

30 X<sup>1</sup> and X<sup>2</sup> are independently -C(O)-, -C(S)-, -C(NR<sub>2</sub>)-, -C(=CN)NH-, -C(O)O-, -C(O)NH-, -C(O)NHSO<sub>2</sub>-, -CH<sub>2</sub>-, -SO<sub>2</sub>-, -P(O)CH<sub>3</sub>-, or an appropriate substituted carbon-nitrogen double bond which links R<sup>3</sup> and/or R<sup>4</sup> to the hydrazino linkage.

37. A formulation according to claim 32 wherein said ecdisone-mimic is N'-(3,5-dimethylbenzoyl)-N-(4-ethylbenzoyl)-N'-(tert-butyl) hydrazine, 5 N,N'-dibenzoyl-N'-(tert-butyl) hydrazine, N'-(3,5-dimethylbenzoyl)-N-(4-ethylbenzoyl)-N'-(tert-butyl)

hydrazine, N'-(3,5-dimethylbenzoyl)-N-(2-methyl-3,4-(ethylenedioxy)-benzoyl)-N'-(tert-butyl) hydrazine, 3,5-di-tert-butyl-4-hydroxy-N-isobutyl-benzamide or 8-O-acetylharpagide.

38. A formulation according to claim 30 wherein said activator for said silent partner is an RXR agonist.

39. A formulation according to claim 38 wherein said RXR agonist is 9-cis-retinoic acid, 4-(1-(3,5,5,8,8-pentamethyl-5,6,7,8-tetrahydro-2-naphthyl)-ethenyl) benzoic acid,

5 ((E)-2-(2-(5,6,7,8-tetra-hydro-3,5,5,8,8-penta-methyl-2-naphthyl)propen-1-yl)-4-thiophenecarboxylic acid, 2-(5,6,7,8-tetra-hydro-5,5,8,8-tetramethyl-2-naphthyl)-2-(carboxyphenyl)-1,3-dioxolane, 4-(5H-2,3-(2,5-dimethyl-2,5-hexano)-5-methyl-10 dibenzo(b,e)(1,4)diazepin-11-yl)-benzoic acid or thiadiazepin analogs thereof, 3,7,11,15-tetramethyl hexadecanoic acid (phytanic acid), 6-(1-(3,5,5,8,8-pentamethyl-5,6,7,8-tetrahydronaphthalen-2-yl)cyclopropyl)nicotinic acid, 2-(4-carboxyphenyl)-2-15 (5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1,3-dithiane or 4-(2-methyl)-1-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)propenyl)benzoic acid.

40. A formulation according to claim 38 wherein said RXR agonist is LG1000268 or LGD 1069.

41. A kit comprising:

at least one activator for a modified ecdysone receptor, wherein said modified ecdysone receptor comprises:

5                   a ligand binding domain capable of binding an  
                  ecdysteroid;  
                  a DNA-binding domain; and  
                  an activation domain,  
                  wherein at least one of said DNA-binding  
10                  domain or said activation domain is not  
                  obtained from a native ecdysone receptor, and  
                  wherein said modified ecdysone receptor  
                  forms a heterodimer with a silent partner  
                  therefor,

15                  at least one activator for said silent partner for said  
                  modified ecdysone receptor, and  
                  a pharmaceutically acceptable carrier.

42. A kit according to claim 41, wherein said DNA binding domain is obtained from a DNA binding protein.

43. A kit according to claim 41 wherein said activator for said modified ecdysone receptor is an ecdysteroid selected from the group consisting of a naturally occurring ecdysone, an ecdysone-analog and an ecdysone-mimic.

Figure 1A

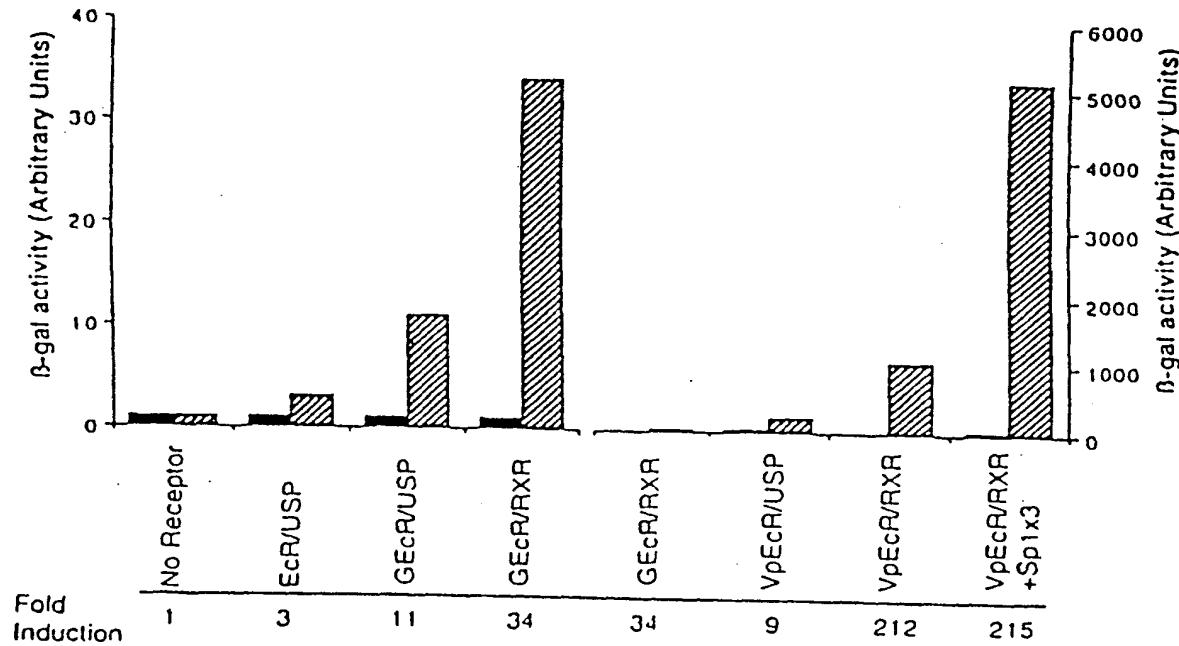


Figure 1B

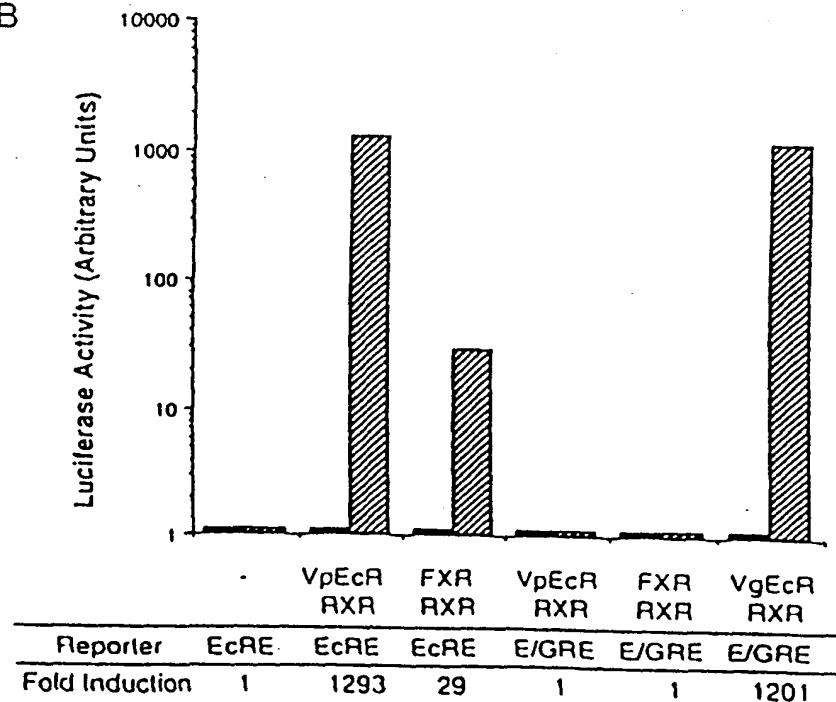


Figure 1C

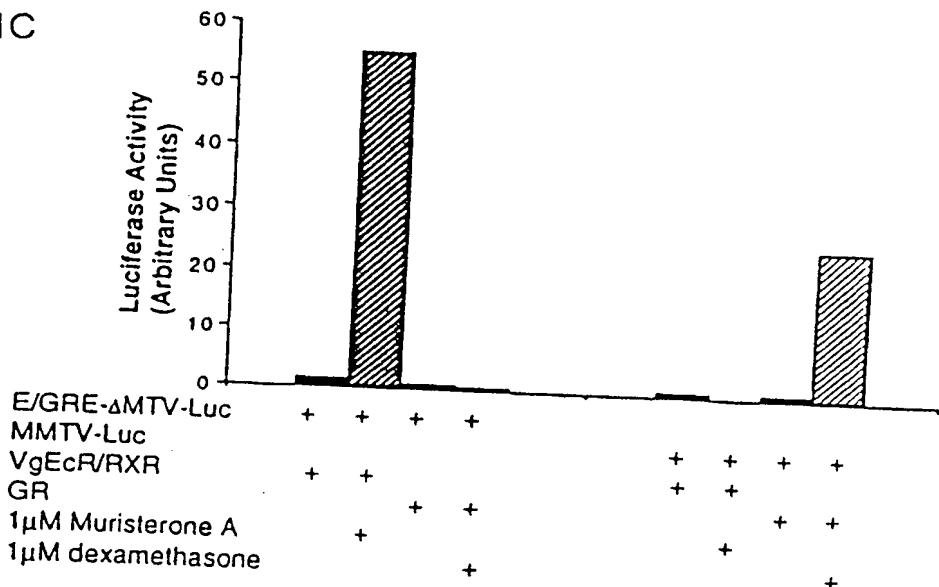
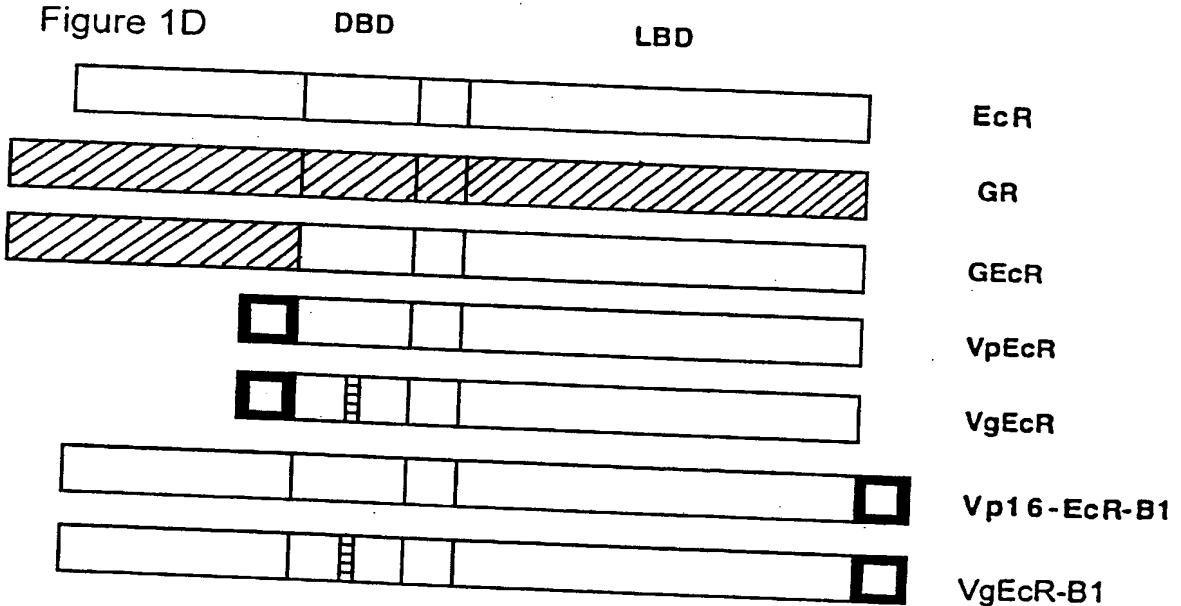


Figure 1D



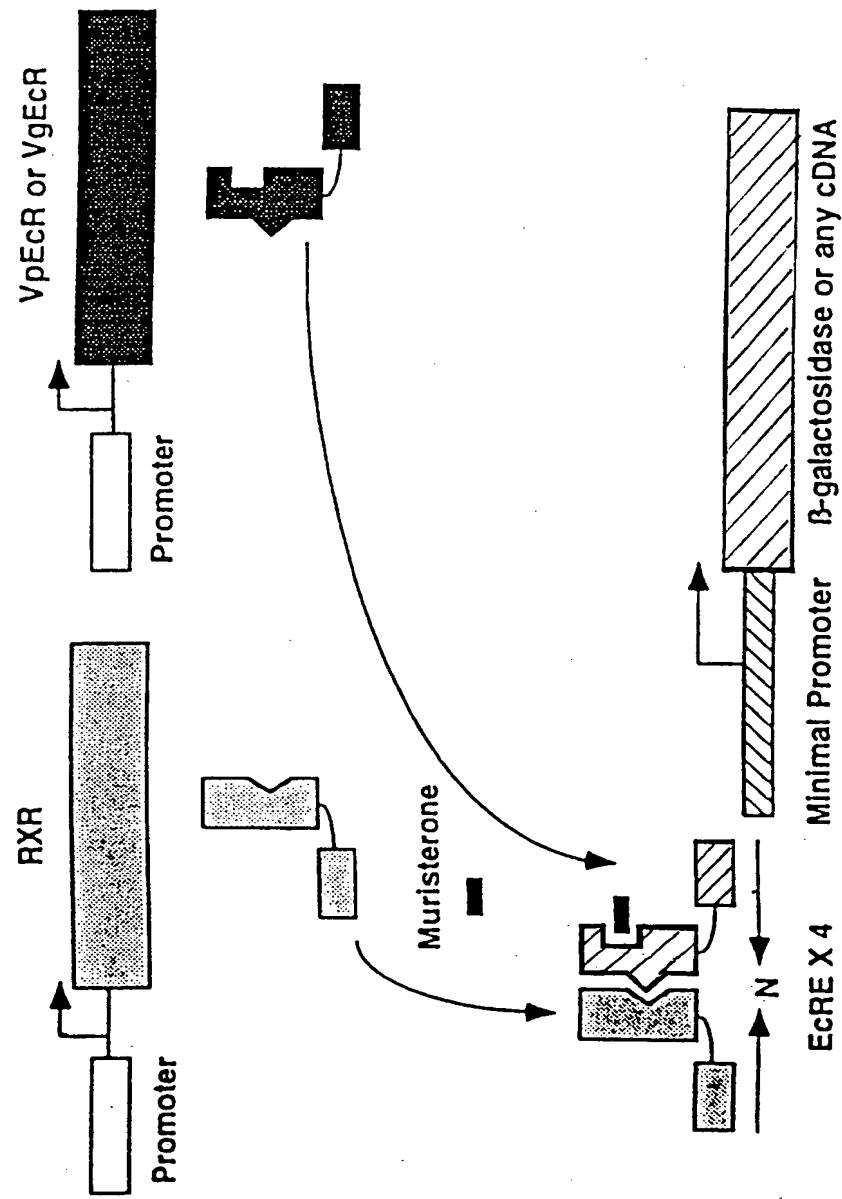
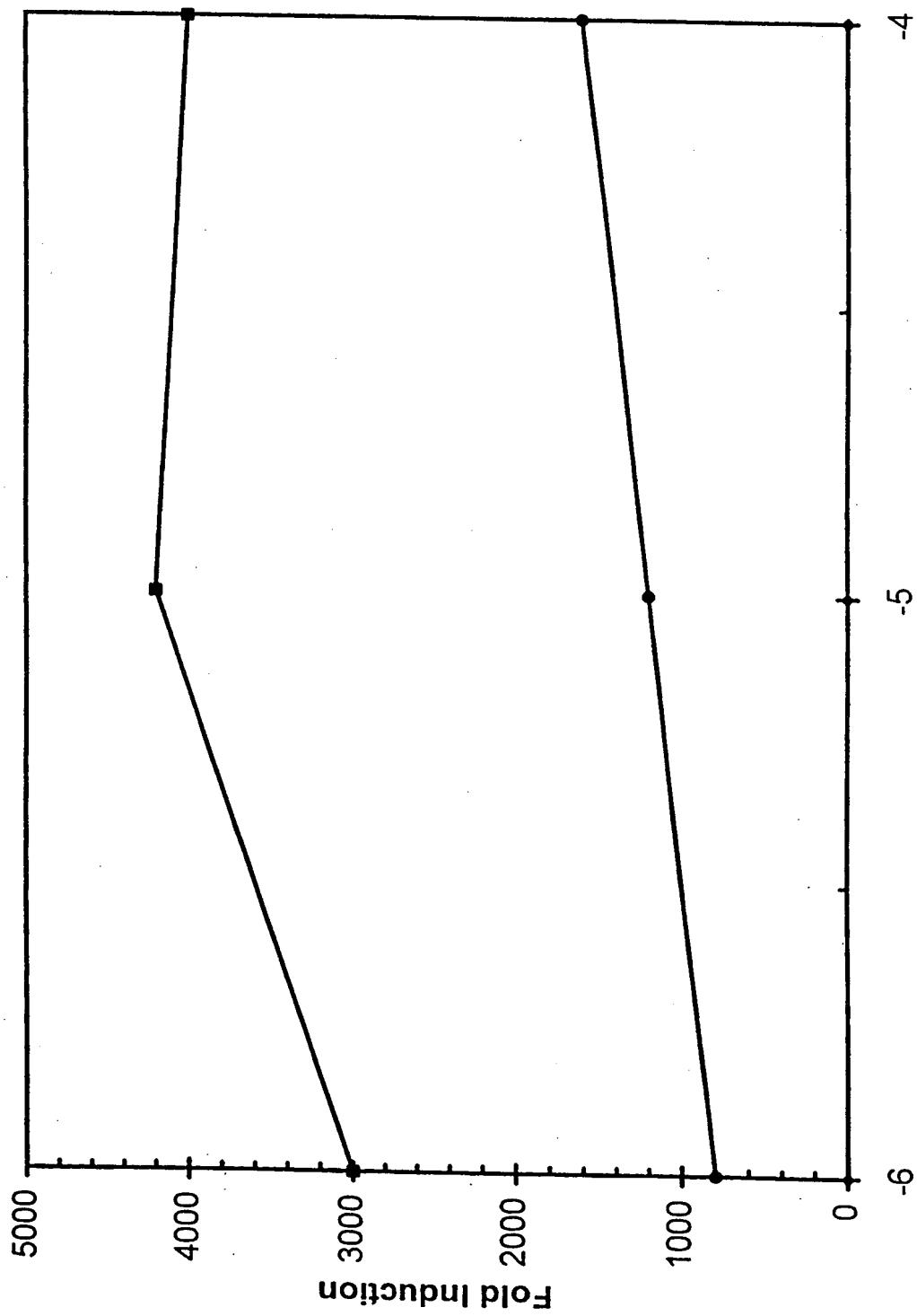


Figure 2

SUBSTITUTE SHEET (RULE 26)



**Figure 3**  
Ligand Concentration

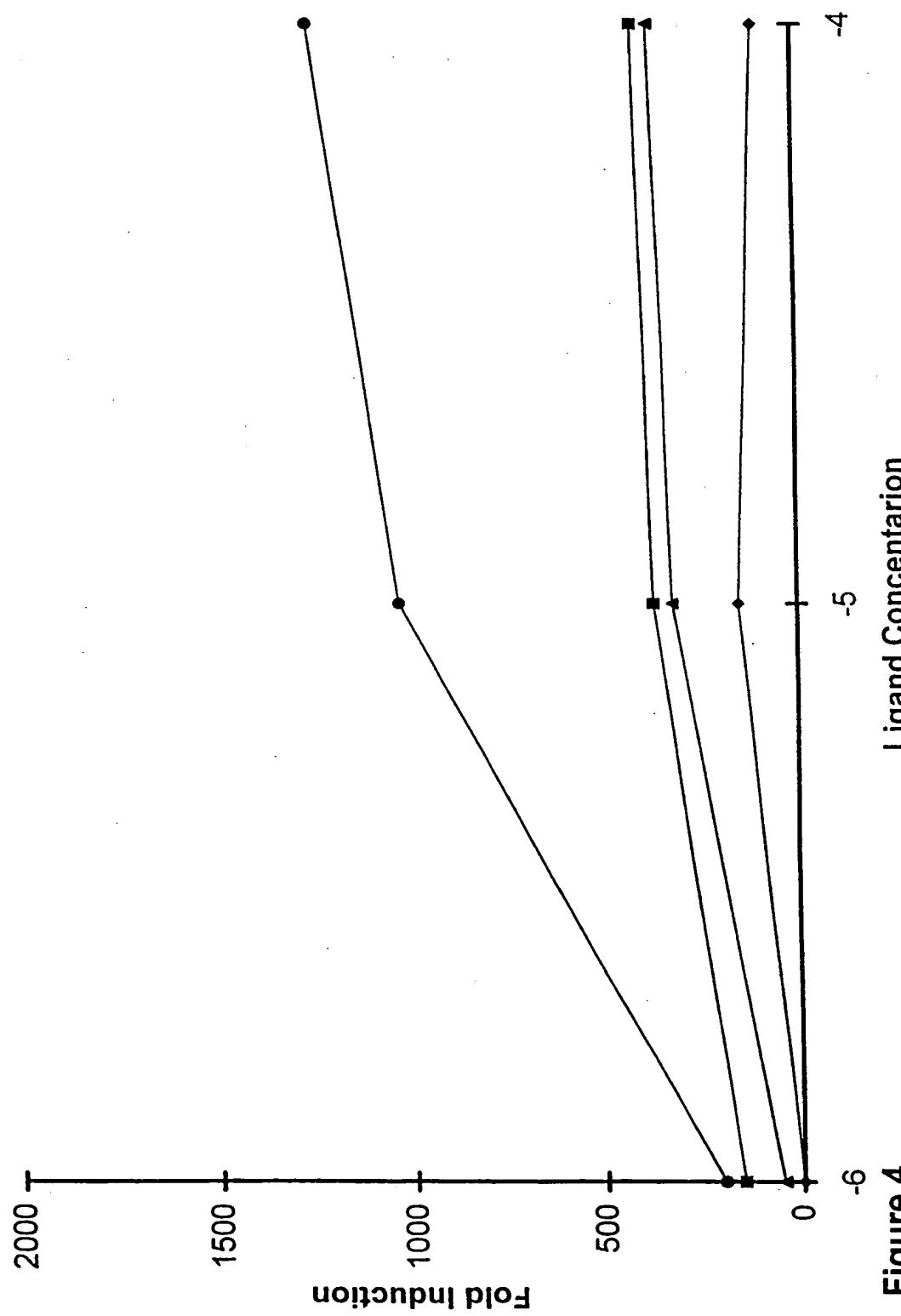


Figure 4

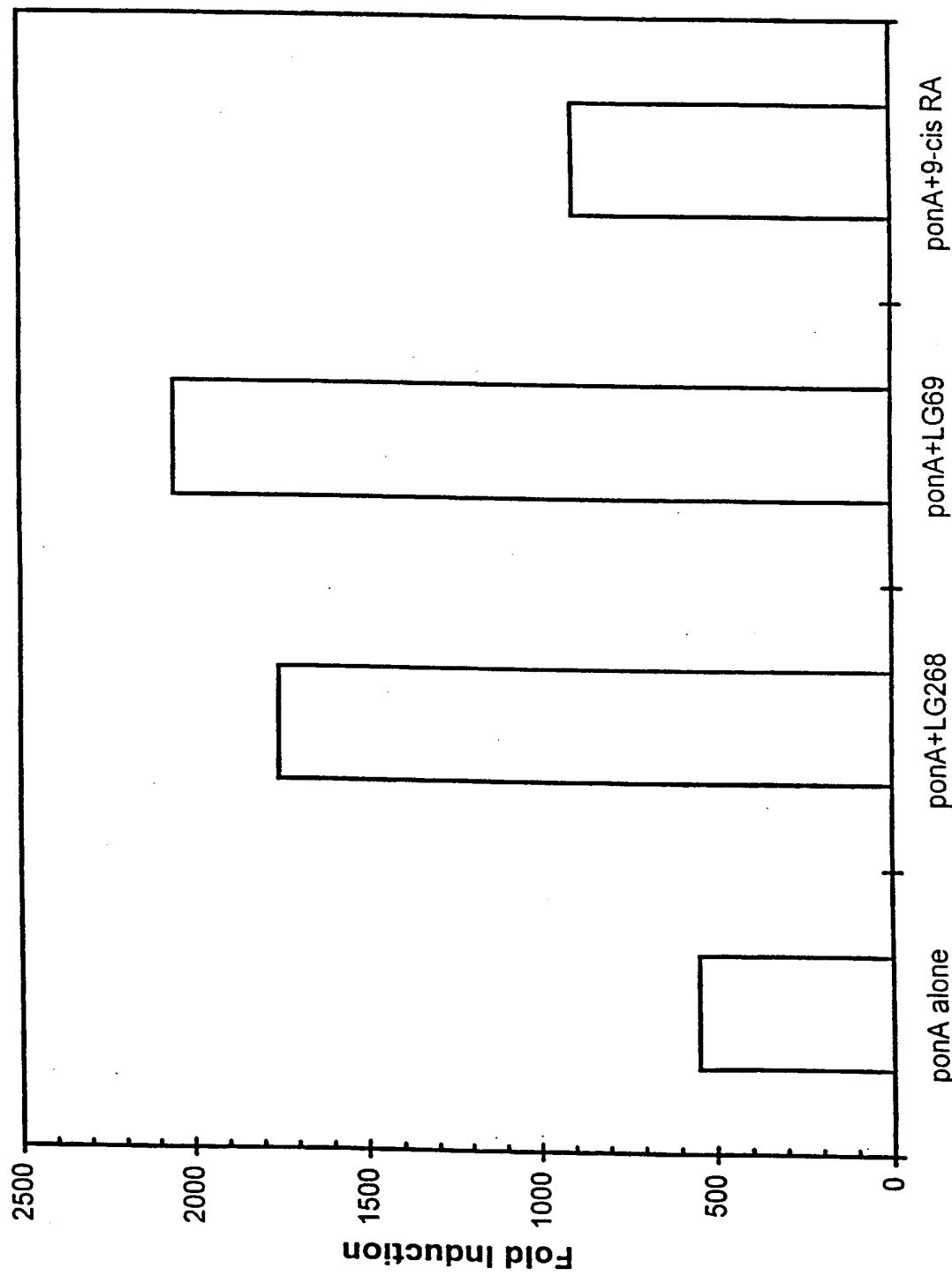


Figure 5

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANT: Evans, Ronald M.  
Saez, Enrique
- (ii) TITLE OF INVENTION: FORMULATIONS USEFUL FOR MODULATING EXPRESSION OF EXOGENOUS GENES IN MAMMALIAN SYSTEMS, AND PRODUCTS RELATED THERETO
- (iii) NUMBER OF SEQUENCES: 12
- (iv) CORRESPONDENCE ADDRESS:
  - (A) ADDRESSEE: Gray Cary Ware & Freidenrich LLP
  - (B) STREET: 4365 Executive Drive, Suite 1600
  - (C) CITY: San Diego
  - (D) STATE: CA
  - (E) COUNTRY: USA
  - (F) ZIP: 92121
- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: Floppy disk
  - (B) COMPUTER: IBM PC compatible
  - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
  - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
  - (A) APPLICATION NUMBER:
  - (B) FILING DATE:
  - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
  - (A) NAME: Reiter, Stephen E.
  - (B) REGISTRATION NUMBER: 31,192
  - (C) REFERENCE/DOCKET NUMBER: SALK2310
- (ix) TELECOMMUNICATION INFORMATION:
  - (A) TELEPHONE: 619-677-1409
  - (B) TELEFAX: 619-677-1409

## (2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 71 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: both
- (ii) MOLECULE TYPE: protein
- (v) FRAGMENT TYPE: internal

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Cys Xaa Xaa Cys Xaa Xaa Asp Xaa Ala Xaa Gly Xaa Tyr Xaa Xaa Xaa  
1 5 10 15

Xaa Cys Xaa Xaa Cys Lys Xaa Phe Phe Xaa Arg Xaa Xaa Xaa Xaa Xaa  
20 25 30

Xaa Xaa Xaa Xaa Xaa Xaa Cys Xaa Xaa Xaa Xaa Xaa Xaa Xaa Cys  
35 40 45

Xaa Xaa Xaa Lys Xaa Xaa Arg Xaa Xaa Cys Xaa Xaa Cys Arg Xaa Xaa  
50 55 60

Lys Cys Xaa Xaa Xaa Gly Met  
65 70

## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Glu Gly Cys Lys Gly  
1 5

## (2) INFORMATION FOR SEQ ID NO:3:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Gly Ser Cys Lys Val  
1 5

## (2) INFORMATION FOR SEQ ID NO:4:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: both

(ii) MOLECULE TYPE: DNA (genomic)

## (ix) FEATURE:

- (A) NAME/KEY: misc\_feature
- (B) LOCATION: 7
- (D) OTHER INFORMATION: /product= "Modified Ecdysone Response Element"  
/note= "N at position 7 is 0 up to 5 nucleotides, with 1 nucleotide being especially preferred."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

RGBNNMNTGN NCY

13

## (2) INFORMATION FOR SEQ ID NO:5:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: both

(ii) MOLECULE TYPE: DNA (genomic)

## (ix) FEATURE:

- (A) NAME/KEY: misc\_feature
- (B) LOCATION: 7
- (D) OTHER INFORMATION: /product= "Modified Ecdysone Response Element"  
/note= "N at position 7 can be 0 up to 5 nucleotides, with 1 nucleotide being preferred."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

RGNNCANKNN VCY

13

## (2) INFORMATION FOR SEQ ID NO:6:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: both

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

AGTGCANTGT TCT

13

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: both

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: misc\_feature
- (B) LOCATION: 7
- (D) OTHER INFORMATION: /product= "Ecdysone Response Element"  
/note= "N at position 7 can be 0 up to 5 nucleotides, with 3 nucleotides being preferred."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

RGBNNNMNRGB NNM

13

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 49 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: both

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

TACAACGCC C TCACCTGTGG ATCCTGCAAG GTGTTCTTT CGACGCAGC

49

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 53 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: both

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GTACTCCCGG GGCGGGGCTA TGCGGGGCGG GGCTAATCGC TAGGGGCAGG GCA

53

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 53 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: both

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GTACTGCCCGG GCCCCTAGCG ATTAGCCCCCG CCCCCGCATAG CCCCCGCCCCCG GGA

53

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 34 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: both

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

AGCTCGATGG ACAAGTGCAT TGTTCTTGC TGAA

34

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 35 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: both

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

AGCTTTCAGC AAGAGAACAA TGCACTTGTC CATCG

35

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US99/08381

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A61K 48/00, 31/33  
US CL : 514/44, 183

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/44, 183

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)  
BIOSIS, EMBASE, MEDLINE, DERWENT BIOTECHNOLOGY, CAS, APS

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 97/45731 A1 (AMERICAN CYANAMID COMPANY) 04 December 1997, see the entire document.	1-43N
Y	WO 94/01558 A2 (THE SALK INSTITUTE FOR BIOLOGICAL STUDIES) 20 January 1994, see the entire document.	1-43
Y	WO 97/38117 A1 (THE SALK INSTITUTE FOR BIOLOGICAL STUDIES) 16 October 1997, see the entire document.	1-43
Y	WO 97/48277 A1 (THE SALK INSTITUTE FOR BIOLOGICAL STUDIES) 24 December 1997, see the entire document.	1-43
Y	WO 96/37609 A1 (ZENECA LIMITED) 28 November 1996, see the entire document.	1-43

Further documents are listed in the continuation of Box C.  See patent family annex.

* Special categories of cited documents:		*T* Later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention	
*A*	document defining the general state of the art which is not considered to be of particular relevance		
*E*	earlier document published on or after the international filing date	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*L*	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*O*	document referring to an oral disclosure, use, exhibition or other means	*A*	document member of the same patent family
*P*	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
11 AUGUST 1999	13 SEP 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer BRUCE CAMPBELL Telephone No. (703) 308-0196

**INTERNATIONAL SEARCH REPORT**

International application No.  
PCT/US99/08381

**C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 96/27673 A1 (CIBA-GEIGY AG) 12 September 1996, see the entire document.	1-43
Y	CHRISTOPHERSON et al. Ecdysteroid-dependent regulation of genes in mammalian cells by a Drosophila ecdysone receptor and chimeric transactivators. Proceedings of the National Academy of Science USA. July 1992, Vol. 89, pages 6314-6318, see the entire document.	1-43
Y	MIKATANI, K. A New Nonsteroidal Chemical Class of Ligand for the Ecdysteroid Receptor 3,5-di-tert-butyl-4-hydroxy-N-isobutyl-benzamide Shows Apparent Insect Molting Hormone Activities at Molecular and Cellular Levels. Biochemical and Biophysical Research Communications. 1996, Vol. 227, pages 427-432, see the entire document.	1-43